Roles of siRNAs and miRNAs in host responses to virus infection: Identification and characterization of a novel viral suppressor of RNA silencing

CHEN JUN

A THESIS SUBMITTED
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
INSTITUTE OF MOLECULAR AND CELL BIOLOGY
NATIONAL UNIVERSITY OF SINGAPORE
2004

Acknowledgements

I gratefully acknowledge the Institute of Molecular Agrobiology and the Institute of Molecular and Cell Biology (both affiliated to National University of Singapore) for their generous financial support that made everything possible. I would like to thank my two supervisors Dr Ding Shou-wei and Dr Peng Jinrong, for their invaluable advice, guidance and encouragement throughout this study. In addition, I would like to give my special thanks to Dr Peng Jinrong for providing me the chance to continue my research project in his lab. I sincerely thank my thesis committee members Associate Professor Wong Sek Man, Associate Professor Zhang Lian-Hui and Dr Yang Wei-cai for their comments and suggestions during my thesis research.

I would like to give my special thanks to Professor Chua Nam Hai for giving us the Amp-TYMV transgenic *Arabidopsis* lines. Sincere thanks to all the members of the former Molecular Virology Laboratory of Institute of Molecular Agrobiology who have rendered me kind help, discussion and advice. They are Wang Shouhai, Guo Huishan, Li Wanxiang, Li Hongwei, Ji Lianghui, Xiao Huogen, Andrew P. Lucy, and Fang Yun. Thanks also go to all members of Functional Genomics laboratory: Lee Sorcheng, Alamgir Hussain, Guo Lin, Huang Hong Hui, Ruan Hua, Cheng Hui, Xu Min, Zhang Zhenhai, Ma Wei Ping, Cheng Wei, Cao Dong Ni, Wen Chao Ming, Fu Check Teen, Lo Leejane and Soo Hui Meng. Special thanks to Liu Fuqian, Fei Jifeng for their help.

I wish to pay special tributes to my parents for their encouragement and understanding. Finally special thanks to my wife, Ms Wu Hua, for her full support and love, and to my son, Chen Yuelin, for his understanding and love.

CONTENTS

Summary

Diverse plant viruses have been found to encode suppressors of post-transcriptional gene silencing (PTGS) since the first reports in 1998. However, few viral suppressors were isolated from viruses that cause diseases in hosts for which the whole genome sequence is available. *Turnip yellow mosaic virus* (TYMV) naturally infects *Brassicaceae* species and is highly pathogenic in *Arabidopsis thaliana.* In this thesis, I describe the identification of the TYMV 69 kDa protein as a viral suppressor of PTGS that exhibits two novel features.

First, p69 suppresses PTGS induced by sense-RNA transgenes but not by transgenes that encode an RNA with potential to fold into double-stranded RNA. p69 suppression of sense-RNA PTGS is associated with the elimination of both siRNA production and DNA methylation, phenocopying genetic mutations in host genes such as the cellular RNA-dependent RNA polymerase (RdRP) involved in the synthesis of the dsRNA trigger. It is concluded that p69 targets at a step in the cellular RdRP pathway that is upstream of dsRNA, rather than downstream of dsRNA as has been suggested for the potato virus X 25 kDa protein.

Second, transgenic *Arabidopsis* plants expressing p69 display disease-like symptoms in absence of TYMV infection. RNA analyses revealed that these plants contained elevated levels of all seven miRNAs examined as well as the mRNA of *Dicer-Like 1* (*DCL1*) required for miRNA production. miRNAs play a regulatory role in the

development of plants and animals by targeting mRNAs for either translational repression or cleavage like siRNAs. As expected, enhanced miRNA-guided cleavage of four cellular mRNAs were detected in p69 transgenic plants. Based on these data I propose that the increase in miRNA abundance results from a negative feedback regulation on *DCL1* triggered by p69 suppression of the RNA silencing antiviral defense and that miRNAs play a pathogenic role in the induction of viral diseases.

CHAPTER 1

LITERATURE REVIEW

1.1 Posttranscriptional gene silencing

1.1.1 Discovery of gene silencing

One of the most remarkable stories in biology over the last decade has been the discovery that an unusual form of RNA can guide silencing of genes in eukaryotes. Gene silencing was first uncovered in the late 1980's during attempts to overexpress transgenes in transgenic plants (Napoli et al., 1990; van der Krol et al., 1990). For example, instead of deep purple flowers as expected, many flowers of the transgenic petunia plants carrying a chalcone synthase (*chs*) transgene, became variegated or virgin white (Napoli et al., 1990). Detailed molecular analysis showed that both transgenic and endogenous *chs* genes were co-suppressed, leading to suppression of entire floral pigment biosynthetic pathway in the white tissue cells. Subsequent work by Dougherty and others demonstrated that a transgene can also be silenced by infection with an RNA virus whose genome shares sequence homology with the transgene and that gene silencing occurs after transcription (Lindbo et al., 1993; Dougherty and Parks, 1995).

Plant researchers were not the only ones getting odd results from their genetic manipulations. Cogoni and Macino (1994) found that transformation of a gene for carotenoid synthesis in the mold *Neurospora crassa* led to inactivation of the endogenous gene in about 30% of the transformed cells. They called this gene inactivation "quelling".

Anomalous results also turned up in experiments in which researchers such as Su Guo and Kenneth Kemphues put antisense RNA into the nematode *Caenorrhabditis*

elegans's cells (Guo and Kemphues, 1995). Not only antisense RNAs blocked production of the protein encoded by the target mRNA, injection of sense RNA in the control experiments also led to similar gene shut-down. In 1998, Fire and colleagues reported that injection of double-stranded RNA (dsRNA) caused much more potent gene silencing in *C. elegans* than either sense or antisense RNAs (Fire et al., 1998). This specific gene silencing induced by dsRNA injection, called RNA interference (RNAi), has since been observed in a number of other organisms, such as flies *Drosophila*, *Tribolium*, *trypanosomes*, Lymnaea, chick, mice and even human cell lines (Tuschl et al., 1999; Brown et al., 1999; Korneev et al., 2002; Hernandez-Hernandez et al., 2001; de Wit et al., 2002; Schwarz et al., 2002). Strong gene silencing was also detected in transgenic plants carrying both sense and antisense transgenes brought together by genetic crosses, which would give rise to dsRNA, suggesting that gene silencing firstly described in transgenic plants may also be induced by dsRNA (Waterhouse et al., 1998; Smith et al., 2000).

Further genetic and molecular evidence confirmed that there were related mechanisms of RNA silencing in both plants and animals. For example, homologous genes were required for RNA silencing in *Neurospora*, *C. elegans* and *Arabidopsis thaliana* (Smardon et al., 2000; Cogoni and Macino, 1999; Dalmay et al., 2001; Dalmay et al., 2000b). Furthermore, small RNAs of 21-25 nucleotides long first detected in silencing plants (Hamilton and Baulcombe, 1999) were also found to be associated with RNAi in other organisms (Hammond et al., 2000; Zamore et al., 2000). The small RNAs, now known as small interference RNAs (siRNAs), also induce specific gene silencing in mammalian cells (Elbashir et al., 2001). Thus, the studies by plant scientists led to the discovery of a completely novel RNA-guided gene regulatory mechanism that is universally conserved among many eukaryotic organisms including mammals.

1.1.2 Mechanism of PTGS

1.1.2.1 Homolog-dependent gene silencing

Transgene-induced silencing effects can be divided into two categories: transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) (Bahramian and Zarbl, 1999; Cogoni and Macino, 1999; Vaucheret and Fagard, 2001). Both TGS and PTGS are nucleotide sequence homology dependent. However, TGS requires homology between promoter regions, and is associated with *de novo* methylation in promoter regions that can be meiotically inheritable (Jones et al., 2001). By contrast genes targeted for PTGS share homology in transcribed regions, and are associated with *de novo* methylation in the transcribed region that will be demethylated during meiosis (Baulcombe, 1999; Chicas and Macino, 2001; Ding, 2000; Fire, 1999; Matzke et al., 2001). Most importantly, TGS silences genes at the level of transcription in the nucleus, whereas PTGS has no apparent effect on transcription of the target gene but promote a rapid and specific degradation of RNA transcripts in the cytoplasm. In addition, PTGS can be systemic silencing (Voinnet and Baulcombe, 1997; Palaqui et al., 1997), but TGS is not involved in systemic silencing (Mlotshwa et al., 2002).

Available evidence shows that PTGS in plants and RNAi in animals and quelling in *Neurospora crassa* represent a highly conserved mechanism, indicating an ancient origin (Vance and Vaucheret, 2001; Cogoni and Macino, 2000; Carthew, 2001; Sharp, 2001; Hutvagner and Zamore, 2002). The core pathway involves a dsRNA that is processed into siRNAs that guide recognition and targeted cleavage of homologous mRNA. dsRNAs that trigger PTGS/RNAi can be made in the nucleus or cytoplasm in a

number of ways, including transcription through inverted DNA repeats, simultaneous synthesis of sense and antisense RNAs, viral RNA replication, and the possible dsRNA synthesis by the activity of cellular RNA-dependent RNA polymerase (RdRP) on single-stranded RNA templates. In *C. elegans*, dsRNAs can be injected or introduced simply by soaking the worms in a solution containing dsRNA or feeding them bacteria expressing sense and antisense RNAs (Plasterk and Ketting, 2000).

1.1.2.2 How does PTGS proceed?

One of the most important approaches applied in the studies of PTGS is genetic screening for PTGS defective mutants. A dozen genes required for PTGS have been identified in *Neurospora*, *Arabidopsis*, *C. elegans* and *Chlamydomonas*, respectively. Significantly, these independent screenings have identified several sets of genes in different organisms that are homologues of each other. The *QDE-1* from *Neurospora* (Cogoni and Macino, 1999), *SDE1/SGS2* from *Arabidopsis* (Dalmay et al., 2000b; Mourrain et al., 2000), and *EGO1*, *RRF-1* from *C. elegans* (Smardon et al., 2000; Sijen et al., 2001), form the first set and proteins encoded by these genes are similar to the tomato RdRP. The proposed role of the cellular RdRP in *Arabidopsis* is to convert an aberrant single-stranded (ss) RNA of a transgene into a dsRNA to trigger PTGS since *SDE1/SGS2* is required for PTGS induced by sense RNA transgenes though not by most RNA viruses tested which encode their own RdRP or by transgenes that encode inverted repeat RNAs (IR-RNAs).

The second set of genes, *QDE-2* from *Neurospora* (Catalanotto et al., 2000), *RDE-1* from *C. elegans* (Tabara et al., 1999), *AGO1, AGO2* from *Drosophila* (Williams and Rubin 2002; Carmell et al., 2002) and *AGO1*, *AGO4* from *Arabidopsis* (Fagard et al., 2000;

Zilberman et al., 2003) belong to the Argonaute family. Argonaute proteins are ~100 kDa, highly contain two common domains PIWI and PAZ. *RDE-1* can interact with *RDE-4*, a dsRNA binding protein, which also can interact with *C. elegans* Dicer homolog-*DCR-1* (RNase III), to initiate RNAi (Parrish and Fire, 2001; Tabara et al., 2002). *RDE-1* is not necessary for gene silencing induced by short antisense RNAs (Tabara et al., 2002). This suggests that *RDE-1* together with *RDE-4* may function to detect foreign dsRNA and to present this dsRNA to *DCR-1* for processing. The function of *AGO1* of *Arabidopsis* seems different. *AGO1* is required for transgene silencing, but not for inverted-repeat induced silencing (Beclin et al., 2002). This suggested that AGO1 may function in recognizing aberrant RNAs, instead of dsRNAs, to help RdRP to synthesize dsRNAs to initiate PTGS. In addition, *AGO1*, which is expressed throughout the plant at all stages of development, was first isolated as a mutant that pleiotropically affects general plant architecture (Fagard et al., 2000). The *ago1* mutants exhibit numerous phenotypic abnormalities such as radicalized leaves, and abnormal infertile flowers. Fertile hypomorphic *ago1* mutants were isolated, which were impaired in PTGS and viral resistance but developmentally close to normal (Morel et al., 2002).

RNA helicase, DNA helicase, RNaseD and dsRNA binding proteins form the third set. The *SDE3* from *Arabidopsis* (Dalmay et al., 2001), *SMG-2* from *C. elegans* (Page et al., 1999), and *MUT-6* from *Chlamydomonas* are homologues to RNA helicase (Wu-Scharf et al., 2000), and were proposed in RNA unwinding. The *QDE-3* from *Neurospora* is a homologue of DNA helicase, and proposed function in the initiation of silencing (Cogoni and Macino, 1999). The *MUT-7* from *C. elegans* is similar to RNaseD, proposed for target RNA degradation (Ketting et al., 1999; Parrish and Fire, 2001). The *RDE-4* from *C. elegans* was identified as a dsRNA binding protein (Parrish and Fire, 2001; Tabara et

al., 2002). It also can bind *DCR-1* and *RDE-1*. It is also not required for short antisense RNAs to induce target gene silencing. Its function may be the same as that of *RDE-1*.

Both *SGS3* and *HEN1* are unique to plants and have no similarity with any known protein (Mourrain et al., 2000; Boutet et al., 2003). There are still a number of genes involved in the PTGS pathway that are being cloned such as SDE4 (Dalmay et al., 2000).

Although genetic studies provided the first clues about the RNA silencing pathway, the most detailed insight on how PTGS proceeds *in vivo* has come from biochemical experiments with *Drosophila* extracts (Tuschl et al., 1999; Hammond et al., 2000; Ketting et al., 2001). The first step involves, Dicer, which is a dsRNA endonuclease (RNase III-like) that processes dsRNA into 21-25 nucleotides dsRNAs (Hammond et al., 2000; Ketting et al., 2001). These small interference RNAs (siRNAs), which were first described in a plant system (Hamilton and Baulcombe, 1999), are generated in *Drosophila* by an RNase III –type protein termed Dicer (Bernstein et al., 2001). Orthologs of Dicer, which contains an ATP-dependent RNA helicase, a PAZ domain, two RNaseIII domains and a dsRNA-binding domain, have been identified in *Arabidopsis* (Park et al., 2002), *C. elegans* (Ketting et al., 2001; Grishok et al., 2001), mammals (Doi et al., 2003), and *Schizosaccharomyces pombe* (Bernstein et al., 2001). The genetic and molecular data from *C. elegans* showed that Dicer was not the only component involved in this step. *RDE4*, a dsRNA binding protein, and *RDE1* function during the initial steps of RNAi to recognize foreign dsRNA and to present this dsRNA to a Dicer homolog (DCR-1) for processing (Tabara et al., 2002).

In the second step, the antisense siRNAs produced by Dicer serve as guides for a different ribonuclease complex, RNA-induced silencing complex (RISC), which cleaves the single-stranded mRNAs that are complementary to the antisense of siRNA (Bernstein

et al., 2001; Nykanen et al., 2001). The first subunit of RISC to be identified was the siRNA, which presumably identifies substrates through Watson-Crick base-pairing (Bernstein et al., 2001; Nykanen et al., 2001). Zamore and colleagues have recently shown that RISC is formed in embryo extracts as a precursor complex of ~250K (Nykanen et al., 2001); this becomes activated upon addition of ATP to form a ~ 100K complex that can cleave substrate mRNAs. Cleavage is apparently endonucleolytic, and occurs approximately in the middle of the region paired with antisense siRNAs. siRNAs are double-stranded duplexes with two-nucleotide 3' overhangs and 5'-phosphate termini, and this configuration is functionally important for incorporation into RISC complexes. However, single-stranded siRNAs should be most effective at seeking mRNA targets, and one intriguing correlation with the transition of RISC zymogens to active enzymes is siRNA unwinding (Tabara et al., 2002). Other subunits of RISC which were co-purified with RISC from *Drosophila* S2 cells are *AGO2*, a member of the Argonaute gene family (Hammond et al., 2001), *dFXR*, a homolog of the Drosophila fragile X mental retardation protein (*FMRP*), and *VIG*, a Vasa intronic gene (Caudy et al., 2002). Tudor staphylococcal nuclease (Tudor-sn) is the first RISC subunit to be identified that contains a recognizable nuclease domain, and could contribute to the degradation observed in RNAi (Caudy et al., 2003). Tudor-SN contains five staphylococcal/micrococcal nuclease domains and is a component of the RISC enzyme in *C. elegans*, *Drosophila* and mammals (Caudy et al., 2003).

Experiments in *C. elegans* suggest that RNAi requires a target RNA copying step by RdRP, without which siRNAs fail to reach sufficient concentration to accomplish target mRNA cleavage (Sijen et al., 2001). Single-stranded RNA oligomers of antisense

polarity can also be potent inducers of gene silencing, in which gene silencing is accomplished by RNA primer extension using the mRNA as template, leading to the synthesis of dsRNA that is subsequently degraded. Genetic studies in plants and fungi demonstrate a clear role for a family of RdRPs in the mechanism of RNA silencing (Dalmay et al., 2000b; Mourrain et al., 2000; Cogoni and Macino 1999). Furthermore, one *Arabidopsis RdRP* homologue, *SDE1/SGS2*, is only required for sense transgene silencing but is dispensable for virus induced gene silencing (VIGS) that viruses encode their own RdRP proteins, and also dispensable for the silencing induced by an inverted-repeat construct which can produce dsRNA after transcription (Dalmay et al., 2000b; Beclin et al., 2002). A high concentration of siRNA may be achieved *in vivo* by copying the target RNA into a new dsRNA, which is then diced into a new crop of siRNAs (Sijen et al., 2001). In this view, exogenous dsRNA does not produce enough siRNA-programmed RISC complexes to accomplish silencing (Hannon, 2002). Instead, the exogenous dsRNA is proposed to be diced into "primary" siRNAs that function as primers for new double-stranded RNA synthesis. Such synthesis is likely to be catalyzed by the RdRP using target mRNA as a template for RNA synthesis. A recent study on the *Neurospora* RdRP *QDE-1* (Makeyev and Bamford, 2002) showed that purified recombinant protein QDE-1, a genetic component of PTGS in *Neurospora*, possesses RNA polymerase activity *in vitro*. The enzyme performs two different reactions on ssRNA templates, synthesizing either extensive RNA chains that form template-length duplexes or ~9-21-mer complementary RNA oligonucleotides scattered along the entire template. *QDE-1* supports both *de novo* and primer-dependent initiation mechanisms (Makeyev and Bamford, 2002).

Although there is strong evidence that RNA silencing phenomena share a common biochemical machinery, there are differences among different organisms. Using

Drosophila embryo lysates *in vitro* and human cell lines *in vivo*, Zamore's lab (Schwarz et al., 2002) provided very strong evidence that siRNAs only guide endonucleolytic cleavage of the target RNA at single sites, but do not serve as random primers to convert mRNA into dsRNAs that are subsequently degraded to generate new siRNAs. This, together with the absence of a clear RdRP homolog in *Drosophila* or mammalian genomic sequences as reported previously (Lipardi et al., 2001), argues that RNAi may proceed without an *RdRP* in these organisms (Schwarz et al., 2002; Stein et al., 2003).

1.1.2.3 Intercellular signaling and amplification of RNA silencing

A remarkable feature of RNA silencing is its ability to act beyond the cells in which it is initiated. Independent experiments in two different laboratories provided direct evidence for a systemic silencing signal (Palauqui et al., 1997; Voinnet and Baulcombe, 1997). In grafting experiments, systemic silencing was transmitted across a graft junction from spontaneously silenced transgenic tobacco rootstocks to isogenic scions that had not silenced spontaneously (Palauqui et al., 1997). Silencing in the scion was specific for the coding sequence that was silenced in the rootstock, demonstrating that the mobile signal is sequence specific. This sequence specificity suggested that the mobile signal is a nucleic acid or includes a nucleic acid. Independent evidence for the involvement of a systemic signal in RNA silencing has come from the demonstration that systemic silencing can be induced in transgenic tobacco species by using infiltration with *Agrobacterium tumefaciens* (agro-infiltration) or particle bombardment to deliver exogenous DNA homologous to the transgene. No *Agrobacterium* or T-DNA could be detected in systemically silenced tissue of agro-infiltrated plants, indicating that the silencing must

have been propagated by means of a mobile signal (Voinnet and Baulcombe, 1997; Voinnet et al., 1998; Palauqui and Balzergue, 1999).

The patterns of systemic silencing suggest that the signal moves both cell-to-cell and through the phloem, mimicking patterns of viral movement through the plants. In 35S promoter driven *GFP* transgenic plants, stomatal guard cells that have lost the plasmodesmatal connections to adjacent cells before induction of systemic silencing do not become silenced, providing evidence that the signal moves cell-to-cell through plasmodesmata (Voinnet et al., 1998). Movement of the signal through the phloem has been most evident from the establishment of systemic silencing along major and minor veins prior to subsequent spread into mesophyll cells. The silencing signal can travel relatively long distances in plants: at least several centimeters as shown by propagation of silencing through leafless grafted spacers that cannot silence because homologous sequences are absent (Palauqui et al., 1997; Voinnet et al., 1998).

Viruses are excluded from meristems after systemic infection of plants (Matthews, 1991), and this is also true for systemic silencing, as extreme meristemic zones of shoots, flowers, and roots retain green fluorescence subsequent to extensive and persistent systemic silencing of *GFP* transgenes (Voinnet et al., 1998). Similarly, silencing is not observed in meristems in *GUS*-silenced plants (Beclin et al., 1998). Recent data indicate that the mobile signals may not be able to enter the meristem as meristem tissue is not competent for silencing (Foster et al., 2002).

One possible role of the silencing signal in plants is anti-viral. The signal would move together with, or in advance of the virus, and mediate silencing of the viral RNA in the newly infected cells. Consequently the infection would progress slowly or would be arrested (Voinnet et al., 2000).

It remains unclear what is the molecular nature of the mobile silencing signal. A recent study shows that there are two classes of siRNAs produced in plants from a silencing green fluorescent protein (GFP) transgene, short (21-22 nt) and long (24-26 nt) size classes (Hamilton et al., 2002). Viral suppressors (will be discussed later) of RNA silencing and mutations in *Arabidopsis* indicate that these two classes of siRNA have different roles. The long siRNA is dispensable for sequence-specific mRNA degradation, but correlates with systemic silencing and methylation of homologous DNA. Conversely, the short siRNA class correlates with mRNA degradation but not with systemic signaling or methylation. This suggests that the long siRNA plays a separate role that is associated with the systemic signaling of RNA silencing and RNA-directed DNA methylation in the nucleus.

Animals may also have a system for amplification and spread of silencing. This is shown most graphically by *C. elegans* (Tabara et al., 1998). The amplification and spread of silencing in *C. elegans* is based on two phenomena. The first is the observation that RNAi can be transported across cell boundaries. Either injecting dsRNA into intestine or feeding worms with *E. coli* expressing the target gene dsRNA, RNAi can spread from the intestine to other somatic tissues and germ lines; Second, RNAi is remarkably long lived and can be inherited for several generations. RNAi is routinely observed not only in the injected animal but also in all of the injected animal's progeny. Accounting for these phenomena requires firstly a system to pass a signal from cell to cell, and secondly a strategy for amplifying the signal.

As mentioned above, in both plants and in *C. elegans*, PTGS or RNAi requires *RdRP* proteins, which could be involved in amplifying the RNA silencing signal. Using a very elegant genetic approach, Hunter and colleagues identified a protein in *C. elegans*

that is required only for systemic silencing (Winston et al., 2002). The *SID-1* gene encodes a transmembrane protein that may act as a channel for import of the silencing signal. Expression of *SID-1* is largely lacking from neuronal cells, perhaps explaining initial observations that *C. elegans* neurons were resistant to systemic RNAi. *SID-1* homologues are absent from *Drosophila*, consistent with a lack of systemic transmission of silencing in flies, but are present in mammals, raising the possibility that some aspects of RNA silencing may act systemically in mammals. Although competent for systemic silencing, plants do not possess *SID-1* homologues, implying that signal transduction in plants is different from that in animals.

1.1.2.4 The role of methylation and chromatin remodeling in PTGS

DNA methylation and chromatin structure have an integral role in TGS (Paszkowski and Whitham, 2001). In this form of silencing, the promoter and sometimes the coding region of the silenced transgenes are densely methylated. Methylation, or methylation-associated chromatin remodeling, of promoter sequences is thought to prevent binding of factors necessary for transcription. The coding sequences of PTGS-inducing transgenes are also frequently found to be methylated. PTGS can be established in plants with defective methyltransferase1 (*met1*), but the silencing becomes impaired during growth, leading to express with the silenced gene in sectors of the plant (Jones et al., 2001). PTGS fails to establish in mutant plants lacking the chromatin remodeling protein *DDM1* (Morel et al., 2000). These results suggest a role for DNA methylation and/or chromatin structure in both establishment and maintenance of PTGS. On the other hand, mutations in genes required for PTGS (for example, *ago1, sde1/sgs2, sgs3, sde3, hen1* and *ago4*) decrease both PTGS and transgene methylation (Fagard et al., 2000;

Dalmay et al., 2000b; Dalmay et al., 2001; Mourrain et al., 2000e; Boutet et al., 2003; Zilberman et al., 2003; Tabara et al., 1999).

The mechanisms of PTGS and TGS may be more common than was previously thought. The recent animal studies also show that there are mechanistic links between PTGS and TGS. In *C. elegans*, *mut-7* and *rde-2* mutations de-repress transgenes that are silenced at the level of transcription by polycomb-dependent mechanism (Tabara et al., 1999; Ketting et al., 1999). Polycomb-group proteins function by organizing chromatin into 'open' or 'close' conformations, creating stable and heritable patterns of gene expression. Recently, Goldstein and his colleagues (Dudley et al., 2002) have found that the polycomb proteins *MES-3, MES-4* and *MES-6* are required for RNAi, at least under some experimental conditions. Mutant worms with knockouts of polycomb genes were deficient in the RNAi response if high levels of dsRNA were injected, but were not deficient in the presence of limiting dsRNA. Furthermore, mutations in piwi, a relative of the RISC component *Argonaute-2*, compromises co-suppression of dispersed transgenes in *Drosophila* at both the posttranscriptional and transcriptional levels (Pal-Bhadra et al., 2002).

One of the most fascinating and least explored responses to dsRNA involves a possible recognition of genomic DNA by derivatives of the silencing trigger, possibly siRNAs. One model suggests that a variant, nuclear RISC carries a chromatin remodeling complex rather than a ribonuclease to its cognate target. Indeed, it has been noted that homologues of Dicer and RISC components are required in the silencing of centromeric repeats in *S. pombe* (Hannon, 2002). It seems therefore that a principal biological function of the RNA silencing machinery may be to form heterochromatic domains in the nucleus

that are crucial for genome organization and stability. Based on genetic and biochemical evidence obtained thus far, a hypothetical model for PTGS is drawn (**Figure 1.1**).

1.1.3 Natural roles of RNA silencing

Several lines of research indicate that RNA silencing is a general antiviral defense mechanism in plants. The first indication came from studies of pathogen-derived resistance (PDR) in plants. In PDR, resistance to a particular virus is engineered by stably transforming plants with a transgene derived from the genome of the virus. Eventually, it became clear that one class of PDR was the result of RNA silencing of the viral transgene. Once RNA silencing of the transgene had been established, all RNAs with homology to the transgene were degraded, including those derived from an infecting virus (Lindbo et al., 1993). Thus, plant viruses could be the target of RNA silencing induced by a transgene. It was also demonstrated that plant viruses could induce RNA silencing. Virus-induced gene silencing (VIGS) can be targeted to either transgenes or endogenous genes (Ruiz et al., 1998).

The idea that RNA silencing is an antiviral defense pathway is strengthened by observation of natural plant-virus interactions. First, plants can recover from certain plant viral infections, and the recovered plants are resistant to secondary infections by either the initial virus or closely related viruses, indicating that the acquired resistance depends on nucleotide sequence similarity (Covey et al., 1997; Ratcliff et al., 1997). Second, many plant viruses encode proteins that suppress RNA silencing, suggesting a coevolution of defense and counterdefense between the host and the invading viruses (Voinnet et al., 1999).

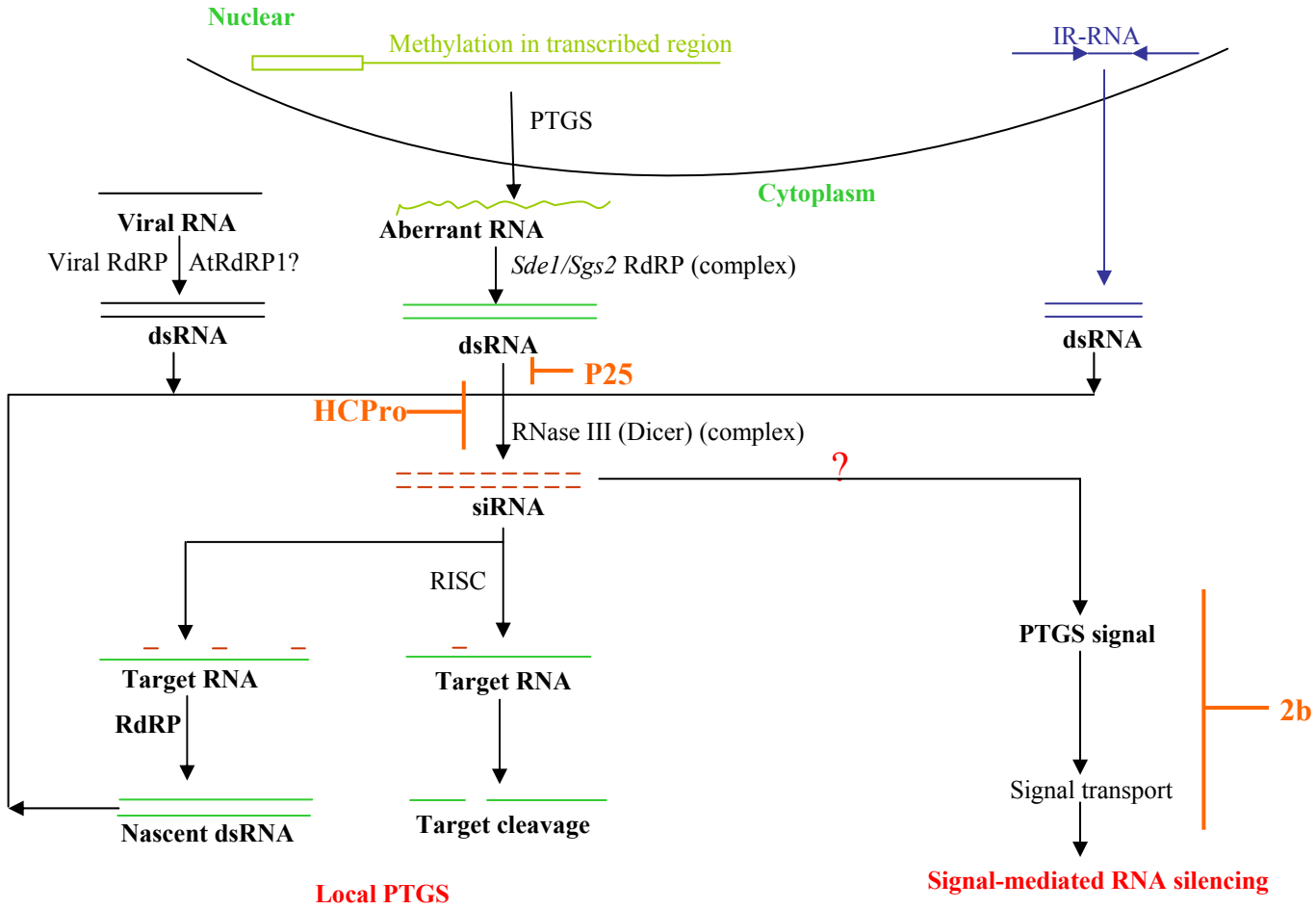


Figure 1.1 Proposed PTGS Model in Plants. dsRNA is proposed to be the common intermediate linking the various ways of initiating RNA silencing. Viruses, as well as transgenes, arranged as inverted repeats, can directly produce dsRNA, whereas transgenes with a single copy sense orientation methylated in transcribed region produce aberrant transcripts that serve as a substrate for producing dsRNA by host RdRP complex. dsRNAs are degraded by Dicer complex into siRNAs. siRNAs will be unwound. Only one strand of siRNAs will incorporate into RISC complex to mediate sequence-specific RNA degradation, or serves as a primer to synthesize nascent dsRNAs, which leads to local PTGS. Longer form (24 nt in length) of siRNAs may be transported systemically to induce signal-mediated RNA silencing.
Plant viral suppressors of PTGS supposedly inhibit PTGS at different steps. HCPro blocks accumulation of siRNAs. 2b inhibits signal-mediated RNA silencing. P25 only inhibits the production of longer form of siRNAs.

Although the *Arabidopsis sgs2/sde1, sgs3* and sde3 mutants were proved to be required for transgene-induced PTGS, surprisingly these mutants exhibited enhanced susceptibility to only Cucumber mosaic virus (CMV) but not to several other viruses (Dalmay et al., 2000i; Mourrain et al., 2000e). Further studies have indicated that these genes may be required only for transgene-specific dsRNA synthesis and not required for initiation of VIGS since viruses contain their own replicases capable of synthesizing dsRNA (Dalmay et al., 2001; Dalmay et al., 2000i; Beclin et al., 2002a). Thus, it is possible that these host genes have no general roles in antiviral defense. A recent study has demonstrated that another *Arabidopsis* RdRP homologue gene-AtRdRP1 plays an important role in antiviral defense (Yu et al., 2003). *AtRdRP1* is induced by salicylic acid treatment and virus infection. An *atRdRP1* knockout mutant accumulated higher and more persistent levels of viral RNAs. These results suggest that one or more of the four *Arabidopsis RdRP* homologs may specifically recognize viral aberrant RNAs. But the mechanism is not clear, as viral siRNA accumulation was not decreased in the *atRdRP1* mutant (Yu et al., 2003).

RNAi also plays a role in viral defense in animals. RNA silencing is an adaptive defense for virus replication in both *Drosophila* and mosquito cell lines (Li et al., 2002; Li et al., 2004). More evidence for this comes from transgenic mosquitoes which were transformed with a fragment of Californinia serogroup virus and that are resistant to the virus replication (Powers et al., 1996). RNAi is becoming a powerful method against human viral and cancer diseases (Aoki et al., 2003; Coburn and Cullen, 2002; Park et al., 2002b; Yamamoto et al., 2002).

A role for the RNA silencing in genome protection is shown by *C. elegans* and *Chlamydomonas* mutants that are defective in RNA silencing and in which there is a high frequency of spontaneous mutation due to enhanced mobility of transposable DNA (Tabara et al., 1999; Ketting et al., 1999; Wu-Scharf et al., 2000). In principle, this genome protection could be targeted through the DICER/RISC process at mRNAs of enzymes required for DNA transposition. However, RNA silencing leads to methylation of the target DNA as well as degradation of the target RNA. The evidence from animal models also shows that there are mechanistic links between PTGS and TGS. [As mentioned in Section 1.1.2.4, in *C. elegans*, *mut-7* and *rde-2* mutations de-repress transgenes that are silenced at the level of transcription by polycomb-dependent mechanism (Dudley et al., 2002; Grishok et al., 2000). Polycomb-group proteins function by organizing chromatin into 'open' or 'close' conformations, creating stable and heritable patterns of gene expression.] These findings indicate that suppression of transposable elements in *C. elegans* and *Chlamydomonas* could be mediated by the effect of RNA silencing on DNA or chromatin.

A role for PTGS pathways in normal regulation of endogenous protein-coding genes was originally suggested through the analysis of plants and animals containing dysfunctional PTGS components. Mutations in the *Argonaute-1* gene of *Arabidopsis*, for example, cause pleiotropic developmental abnormalities that are consistent with alterations in stem-cell fate determination (Fagard et al., 2000; Carmell et al., 2002). A hypomorphic mutation in *Dcl1* causes defects in leaf development and overproliferation of floral meristems (Schauer et al., 2002; Jacobsen et al., 1999). Mutations in Argonaute family members in *Drosophila* also impact normal development. In particular, mutations in *Argonaute-1* have drastic effects on neuronal development, and piwi mutants have

defects in both germline stem-cell proliferation and maintenance (Fagard et al., 2000; Morel et al., 2002). A possible mechanism underlying the regulation of endogenous genes by the PTGS machinery emerged from the study of *C. elegans* containing mutations in their single *Dicer* gene, *Dcr-1* (Knight and Bass, 2001; Grishok et al., 2001). Now it becomes clear that Dicer is also responsible for the production of microRNAs that control development, which will be discussed at Section 1.3.

1.2 Viral suppressors of RNA silencing

An important milestone in the research of PTGS was the discovery in 1998 that plant viruses encode proteins that are suppressors of PTGS (Anandalakshmi et al., 1998; Brigneti et al., 1998; Kasschau and Carrington, 1998). Most of these proteins were previously identified as pathogenic determinants and the first suppressors identified potyviral HC-Pro and CMV 2b both involved in the determination of virus synergy (Ding et al., 1996; Pruss et al., 1997). The discovery of viral suppressors of RNA silencing provided not only the strongest support that PTGS functions as a natural defense against viruses, but also yielded valuable tools to study the molecular mechanism of PTGS. A number of approaches have been used in the identification and mechanistic analysis of viral suppressors of RNA silencing.

Firstly, a plant line carrying a constitutively silenced reporter transgene (such as *GUS*) (Elmayan and Vaucheret, 1996) is either cross-pollinated with a transgenic line containing the suppressor candidate gene, or infected persistently and systemically with a replicating virus vector (e.g Potato Virus X, PVX) which expresses a suppressor candidate gene (Anandalakshmi et al., 1998, Llave et al., 2000; Mallory et al., 2001a; Guo and Ding,

2002). Reactivation of the silenced reporter gene will identify the candidate gene as a suppressor of PTGS. In the second approach, the reporter gene (*GFP* or *GUS*) in a transgenic line can be silenced by leaf infiltration of *Agrobacterium tumefaciens* carrying the same reporter gene cloned within a Ti plasmid, referred to as agro-infiltration (Voinnet and Baulcombe, 1997; Voinnet et al., 2000d; Llave et al., 2000; Guo and Ding, 2002). To assay for silencing suppression in this system, the suppressor can be delivered either before or after the transgene is silenced. In the reversal of silencing assay, recombinant PVX carrying a suppressor is used to infect the transgenic plant after the transgene is systemically silenced. By contrast, in the co-infiltration assay, both inducer and suppressor of RNA silencing are co-introduced into the leaves by agro-infiltration. As a result, expression of the suppressor protein is transient and localized in the co-infiltration assay. A viral protein may suppress local or systemic silencing and both activities can be determined using agro-infltration as in the first approach.

The known viral suppressors of RNA silencing can be broadly divided into the following three groups (Li and Ding, 2001).

1.2.1 The first group

HC-Pro, P1 and AC2 encoded by potyviruses, rice yellow mottle sobemovirus and African cassava mosaic geminivirus, respectively were able to activate *GFP* expression in all tissues of the previously silenced *GFP* plants (Voinnet et al., 1999; Brigneti et al., 1998). Further work showed that transient expression of HC-Pro by agro-infiltration is sufficient to inhibit RNA silencing of a *GUS* transgene in *N. tabucum*. Interestingly, suppression of RNA silencing by HC-Pro was associated with a significantly reduced

accumulation of 25 nt RNAs, but did not prevent production and systemic signaling (Mallory et al., 2001a; Mallory et al., 2002). These data suggest that HC-Pro targets a maintenance step of the RNA silencing pathway that is upstream to the production of the 25 nt RNAs but downstream to the signal production. Hc-Pro suppresses not only sense-transgene silencing, but also RNA silencing induced by inverted-repeat transgenes and viral amplicon-transgenes (Mallory et al., 2002). HC-Pro suppression of silencing induced by inverted-repeat and amplicon transgens was accompanied by the apparent accumulation of long dsRNAs and proportional amounts of the larger class of siRNAs. Thus, HC-Pro may interfere with silencing either by inhibiting siRNA processing from dsRNA precursors or by destabilizing siRNAs (Mallory AC et al., 2002), the latter of which is supported by the recent finding that HC-Pro inhibits miRNA-mediated cleavage of mRNAs (Kasschau et al., 2003)

HC-Pro suppression of RNA silencing may involve protein-protein interaction with a calmodulin-related protein (*rgs-CaM*), over-expression of which also results in silencing suppression in a manner similar to that by HC-Pro (Anandalakshmi et al., 2000).

1.2.2 The second group

2b of CMV was found to produce a distinct silencing suppression pattern in the same silencing reversal assay used for HC-Pro. Expression of Cmv2b from either its own or the PVX genome resulted in *GFP* expression in those leaves that had newly emerged from the growing points, but not in the older tissues in which RNA silencing had already been established before virus infection (Brigneti et al., 1998). The introduction of either Tav2b encoded by tomato aspermy cucumovirus, p19 encoded by tomato bushy stunt

tombusvirus, or CP of turnip crinkle virus into the silenced *GFP* plants produced a similar suppression pattern (Li et al., 1999; Voinnet et al., 1999; Qu et al., 2003). Thus, in contrast to HC-Pro/P1/AC2, this second type of viral suppressor is not able to reverse RNA silencing once silencing is established, indicating they target an earlier stage of RNA silencing than HC-Pro. Further studies demonstrate that Cmv2b encodes a functional nuclear localization signal and nuclear targeting is crucial to its suppression activity in the silencing reversal assay (Lucy et al., 2000). Recent findings using a three-way grafting experiment showed that Cmv2b inhibits the long range signaling activity of the gene silencing signal (Guo and Ding, 2002). A segment of Cmv2b transgenic plant was used as the middle insert between a *GUS*-silencing rootstock and a *GUS*-expressing reporter scion. It was found that the expression of the *GUS* gene in the *GUS*-expressing scion was not affected, suggesting that Cmv2b blocks the transmission of silencing signal generated from the *GUS*-silencing rootstock. Furthermore, Cmv2b also reduces transgene DNA methylation in nucleus.

A study of p19 showed that p19 binds PTGS-generated siRNAs (Silhavy et al., 2002). It suggested that p19 binding of siRNAs may play a role in silencing suppression by sequestering the specificity determinants of the RISC complex. The crystal structures of p19 both from tomato bushy stunt tombusvirus and Carnation Italian ringspot virus showed that p19 proteins act as a molecular caliper to specifically select siRNAs based on the length of the duplex region of the RNA (Ye et al., 2003; Vargason et al., 2003).

1.2.3 The third group

The 25 kDa protein (p25) of PVX displays no detectable suppressor activity in silencing reversal assay, which may explain why PVX is an efficient vector for VIGS (Voinnet et al., 2000). However, systemic RNA silencing did not occur in the majority of the transgenic GFP plants co-infiltrated with 35S-25K, which encodes p25, and 35S-GFP or 35S-PVX:GFP, unlike those infiltrated with 35S-GFP or 35S-PVX:GFP alone. This work clearly demonstrates, for the first time, a role for a viral protein in interfering with the systemic signaling of RNA silencing. Interestingly, although p25 arrested systemic RNA silencing induced by either inducer, it inhibited localized RNA silencing induced by the 35S-GFP transgene but not by the replicating virus 35S-PVX:GFP. This suggests that replication of the PVX RNA genome in *N. benthamiana* plants triggers two independent branches of RNA silencing: one branch is p25-insensitive and the other is similar to the transgene-induced RNA silencing that leads to production of the systemic silencing signal and that is sensitive to p25. As both local and systemic transgene RNA silencing are inhibited by p25, p25 targets a step either at or upstream to the signal production. A recent work suggests that p25 acts downstream of dsRNA by a specific inhibition of the production of the longer siRNA species of 24-26 nt (Hamilton et al., 2002).

In plants, RNA silencing can be induced locally and then spread throughout the organism, and this aspect of the process likely reflects its role in viral defense and suppressor role in counterdefense. Plant viruses generally enter a cell at a small wound, replicate within that cell, and then move cell-to-cell until they reach the vascular tissue, which serves as a conduit to all parts of the plant. The movement of the mobile silencing signal in the plants parallels that of the virus, traveling in the vascular tissue and spreading out from the veins. Thus, an invading virus enters into a race with the host. If the virus

moves faster, it can establish a systemic infection. If the silencing signal goes faster, then the virus will enter systemic tissues only to find RNA silencing already established, and the infection will be halted. This is probably why most of the viral suppressors were originally described to be required for the viral long distance movement. Notably, neither HC-Pro nor Cmv2b interfere with transcriptional gene silencing (Mette et al., 2001).

1.2.4 Animal viral suppressors

Both PTGS and RNAi are manifestations of a broader group of posttranscriptional gene silencing phenomena common to virtually all eukaryotes. PTGS has been demonstrated as a natural antiviral defense in plants. Is RNAi also antiviral in animals? Flock house virus (FHV), which can infect both animals and plants, was demonstrated as both an initiator and a target of RNA silencing in *Drosophila* cells (Li et al., 2002). FHV infection requires suppression of RNA silencing by an FHV-encoded protein, B2. RNA replication of Nodamura Virus (NOV), which is closely related to FHV, also triggers RNA silencing in *Drosophila* and mosquito cells and requires suppression of the antiviral defense by B2 of either FHV or NOV (Li et al., 2004). These findings establish RNA silencing as an adaptive antiviral defense in invertebrate cells. B2 also inhibits RNA silencing in transgenic plants, providing evidence for a conserved RNA silencing pathway in the plant and animal kingdoms. Recent work further showed that vaccinia virus and human influenza A, B, and C viruses each encode an essential protein that suppresses RNA silencing-based antiviral response in cultured *Drosophila* cells (Li et al., 2004). The vaccinia and influenza viral suppressors, E3L and NS1, are distinct dsRNA-binding proteins and essential for pathogenesis by inhibiting the mammalian interferon-regulated

innate antiviral response. It was also demonstrated that the dsRNA-binding domain of NS1, implicated in innate immunity suppression, was both essential and sufficient for RNA silencing-based antiviral response suppression. These findings suggest a possible antiviral role for RNAi in vertebrates.

1.3 microRNAs

1.3.1 Discovery of miRNAs

A growing body of evidence suggests that 21~24-nt noncoding RNA molecules play crucial roles as regulators of gene expression in eukaryotes (Ruvkun, 2001; Jones, 2002; Banerjee and Slack, 2002). The first miRNA genes discovered were *C. elegans lin-4* and *let-7*, which were identified from the genetic analysis of developmental timing in the nematodes. The *lin-4* or *let-7* mutant worms fail to execute certain transition between larval stages. The cloning of first *lin-4* (Lee et al., 1993) and later *let-7* (Reinhart et al., 2000) revealed that these two genes are particularly deviant-unusual small, encoding no protein products, and producing exceedingly short (~22 nt) transcripts from characteristic hairpin RNA precursors about 70 nt long. These small RNAs originally were called small temporal RNA or stRNA. The 22 nt *lin-4* and 21 nt *let-7* RNAs are translational repressors of mRNAs that encode proteins of the heterochronic developmental timing pathway of the worms. *lin-4* RNA is complementary to the sequences in the 3'-untranslated region (UTR) of *lin-14* and *lin-28* mRNAs, and *let-7* RNA is complementary to the 3'-UTR of *lin-14*, *lin-28*, *lin-41*, *lin-42* and *daf-12*. Protein synthesis from these genes is repressed by *lin-4* and *let-7* during the early larval stages of *C. elegans* development to cause the proper sequence of stage-specific developmental events.

When first described, *lin-4* and *let-7* seemed to be unique, since no similar tiny regulatory RNA had been encountered in other organisms. However, *let-7* RNA is phylogenetically conserved in size and nucleotide sequence in essentially all the bilaterally symmetric animals (Pasquinelli et al., 2000). Moreover, *let-7* has a similar developmental profile in diverse taxa, suggesting the conservation of an ancient developmental timing pathway. Indeed, homologs of the worm *let-7* target, *lin-41*, can be found in insects and vertebrates with their *let-7* complementary sites intact. These findings indicated that the *lin-4* and *let-7* class of regulatory genes was not just a worm oddity, and likely represents a gene family that has evolved from an ancient ancestral small RNA gene. Furthermore, stRNAs are similar in size to siRNA, which are a central component of PTGS pathway. PTGS pathway is an evolutionarily conserved genetic surveillance mechanism that can degrade an mRNA in response to the presence of dsRNA corresponding to the targeted mRNA. *lin-4* and *let-7* are not siRNA as they do not trigger degradation of their targets, but the ubiquity of siRNAs suggested that stRNAs have been part of the eukaryotes for a very long time and may have something in common with PTGS pathway. Indeed, the *lin-4* and *let-7* stRNAs are processed from their stem-loop precursor transcripts by the same enzyme, *Dicer*, that generates the ~21nt siRNAs from a dsRNA trigger (Grishok et al., 2001; Hutvagner et al., 2001; Bernstein et al., 2001; Ketting et al., 2001). Since *Dicer* is phylogenetically widespread, stRNA genes could also be commonplace. This also can explain that Dicer mutant *dcr-1* from *C. elegans* and *dcl1* from *Arabidopsis* both exhibit pleiotropic developmental defects, unlike most other PTGS-deficient worm and plant mutants.

1.3.2 Cloning and characterization of miRNAs.

Because stRNAs are noncoding, traditional computational gene finding methods tuned to protein coding potential would miss them, and they would not be represented in conventional cDNA libraries prepared from polyadenylated mRNA. PTGS is one of the hottest areas in biological sciences in recent years. Methods have been developed to clone cDNAs corresponding to the ~21 nt siRNAs produced during PTGS (Elbashir et al., 2001). These methods were adopted to the preparation of endogenous cDNAs corresponding to size-selected (~21 nt) RNAs expressed in worms, flies and human cells (Lagos-Quintana et al., 2001; Lee and Ambros, 2001a; Lau et al., 2001). From these cDNA libraries, more than 150 of sequences corresponding to novel transcripts of about 21 nt were identified and dubbed "microRNAs". The majority of the genes that produce these transcripts are located in intergenic regions. Longer (~70 nt) precursors were identified for these miRNAs and the precursors are predicted to form a hairpin reminiscent of the *lin-4* and *let-7* precursors.

A significant fraction of the miRNA genes seems to be very well conserved phylogenetically. Of the 62 *C. elegans* miRNA genes described so far, 9 are conserved in *Drosophila*, and 7 are conserved in *Homo sapiens*. For these evolutionarily related miRNAs, the sequence of ~21 nt mature miRNA shows the greatest conservation. Such highly conserved sequence in the miRNA presumably reflects complementarity to multiple conserved target sequences. In some cases, the miRNA and antisense targets could be involved in similar pathways across diverse evolutionary distances, as seems to be the case for *let-7*.

The discovery of miRNAs in animal systems prompted three research groups to search for miRNAs in *Arabidopsis*. To date, this approach has been rewarded by discovery of at least 100 plant miRNAs derived from predominantly intergenic locations (Llave et al., 2002; Reinhart et al., 2002; Park et al., 2002a). Only a small proportion of miRNAs was identified many times, indicating that the search has not been saturated. The researchers used RNA-folding programs to analyze the structure of putative transcripts from which the miRNAs could be derived. In the majority of cases, a stem-loop RNA structure was predicted and the miRNAs could correspond to either one of the arms of the stem portion. Northern analysis showed that the expressions of some of the identified miRNAs are subjected to spatial or temporal control. However, as yet, regulatory elements directing transcription of the miRNA precursor transcripts have not been identified, and the majority of miRNA precursors could not be detected.

The availability of the complete genomic sequences of *Arabidopsis* and rice is the key for predicting the identity of the stem-loop precursor RNAs. However, miRNAs are not unique to *Arabidopsis*: researchers have been able to detect by hybridization, miRNAs in maize and tobacco that correspond to several of the *Arabidopsis* miRNAs (Llave et al., 2002; Reinhart et al., 2002; Park et al., 2002a). Reassuringly, most of the rice miRNA homologues are flanked by sequences that have the potential to form stem-loop precursors. Although the flanking sequences surrounding the rice and *Arabidopsis* miRNAs are diverged, the overall duplex structure is conserved, suggesting strong selective pressure (Llave et al., 2002; Reinhart et al., 2002; Park et al., 2002).

1.3.3 Putative targets of miRNAs

The developmental defects of *dcl1* and *hen1* mutants might be a consequence of miss-expression of genes that are usually regulated by miRNAs. This prediction naturally leads to the key questions: what are the regulatory targets of the miRNAs and by which mechanisms do miRNA act to control gene expression? The target mRNAs of the *C. elegans let-7* and *lin-4* miRNAs were identified using genetic techniques. These miRNAs do not perfectly match their targets and consequently it was expected that identification of targets by computational methods would be problematic owing to these imperfect matches. From more than 100 plant miRNAs identified, one miRNA derived from an intergenic region was found to also share perfect anti-sense complementarity with mRNAs encoding for three Scarecrow-like (SCL) transcription factors (Reinhart et al., 2002; Llave et al., 2002b). Because only one of the plant miRNAs identified to date shares perfect complementary with its target, David Bartel's group used computational methods to identify putative mismatched targets to 16 of the plant miRNAs (Rhoades et al., 2002). Randomized sequences of these miRNAs were also used in the searches to give an indication as to the likelihood of finding a mismatched 'hit' by chance. Allowing for three or fewer mismatches, the number of hits to the miRNAs was significantly higher than to the randomized sequences. In total, 49 potential regulatory targets were identified for 14 out of 16 miRNAs examined. Some miRNAs are complementary to more than one mRNA, and for these, all the target mRNAs are members of the same gene family. In many cases, the miRNA complementary sites are conserved between *Arabidopsis* and rice, thus strengthening the likelihood that functionally significant target sites have been identified. So what do the target mRNAs encode for? Intriguingly 34 out of the 49 targets correspond to known or putative transcription factors, many of which are implicated in the control of meristem identity. Thus, a wonderful link can be drawn between the developmental

defects of the *dcl1* and *hen1* mutants, the failure to accumulate miRNAs in those plants and the predicted miRNA targets. More recently discovered miRNA functions, which are involved in the control of leaf and flower development in plants, confirmed the miRNA target prediction in plants (Aukerman and Sakai, 2003; Chen, 2003; Emery, et al., 2003; Palatnik, et al., 2003).

Computational methods have recently been developed to identify the targets of *Drosophila* and mammalian miRNAs (Enright et al., 2003; Lewis et al., 2003; Stark et al., 2003). These methods search for multiple consered regions of miRNA complementarity within 3' UTRs. Identifying targets in animals has been a more difficult task than in plants because in animals there are fewer mRNAs with near-perfect complementarity to miRNAs. This makes the analysis noisier- much more prone to false positives. Furthermore, evolutionary conservation was used as a criterion for target identification in animals, and thus it could not be used as a means to indepentently validate the targets. The experimental support achieved for a majority of the predictions tested is encouraging. In the mammalian studies, over 400 regulatory targets were predicted by identifying mRNAs with conserved pairing to the 5' region of the miRNA (the 7 nt core segment comprising residues 2-8 of the miRNAs) and evaluating the number and quality of these complementary sites. Eleven predicted regulatory targets (out of 15 tested) were supported experimentally using a HeLa cell reporter system (Lewis et al., 2003). The predicted regulatory targets of mammalian miRNAs were enriched for genes involved in transcriptional regulation but also encompassed a broad range of other functions (Lewis et al., 2003).

1.3.4 Biogenesis of miRNA

A 693 bp genomic fragment rescues the *lin-4* deficiency, implying that all the elements required for the regulation and initiation of transcription are located in this short fragment (Lee et al., 1993). However, little is known regarding the transcriptional processes for *lin-4* or any other miRNA genes. Some miRNAs residing in introns are likely to share their regulatory elements and primary transcript with their pre-mRNA host genes. The remaining miRNA genes are presumably transcribed from their own promoters. These primary miRNA transcripts, called pri-miRNAs (Lee et al., 2002), are generally thought to be much longer than the conserved stem loops currently used to define miRNA genes. The current model for maturation of the mammalian miRNAs is as follows: the first step is the nuclear cleavage of the pri-miRNA, which liberates a ~60-70 nt stem loop intermediate, known as the miRNA precursor, or the pre-miRNA (Lee et al., 2002; Zeng and Cullen, 2003). This first processing step is performed by the Drosha RNase III endonuclease, which cleaves both strands of stem at sites near the base of the primary stem loop (Lee et al., 2003). This pre-miRNA is actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Exportin-5 (Yi et al., 2003; Lund et al., 2004).

The nuclear cleavage by Drosha defines one end of the mature miRNA. The other end is processed in the cytoplasm by the enzyme Dicer (Lee et al., 2003). Using a genetic approach, Dicer has been already identified as a key component for miRNA processing in *C. elegans*, *Drosophila*, human cells and *Arabidopsis* (Grishok et al., 2001; Ketting et al., 2001; Hutvagner and Zamore, 2002; Park et al., 2002; Reinhart et al., 2002). In *C. elegans*, *DCR-1* is required for both RNAi and the maturation of the *lin-4* and *let-7* stRNAs (Grishok et al., 2001; Ketting et al., 2001). Unlike most other PTGS-deficient worms, *dcr-*

1 was neither normal nor fertile, caused retarded heterochronic defects similar to *lin-4* and *let-7* mutations. Interestingly, while two Argonaute proteins (AGL-1 and AGL-2) are also involved in the maturation of *lin-4* and *let-7*, they are not required in RNA silencing. Another Argonaute protein RDE1 acts in an opposite way (Grishok et al., 2001), suggesting a partial overlap between the siRNA and miRNA pathways in *C. elegans*.

This stepwise scenario for miRNA maturation is based primarily on the investigation of mammalian Drosha and Dicer function (Lee et al., 2002, 2003). The notion that it applies to other metazoan species is supported by the identity of the long form of the *C. elegans lin-4* RNA, which appears to be an exellent match to that expected for the *lin-4* pre-miRNA (Lee et al., 1993). Furthermore, putative pre-miRNAs for numerous miRNAs can be detected on Northern blots, and when examined in the context of reduced Dicer activity, these pre-miRNAs invariably increase in abundance, suggesting that Dicer is responsible for their processing (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Lee and Ambros, 2001; Lim et al., 2003).

The cloning of a few miRNA pairs that are complementary to each other points to a transient miRNA:miRNA* duplex similar to siRNA (Reinhart et al., 2002). However, the biogenesis of this duplex appears to differ in plants. Most notably, pre-miRNAs have not been compellingly detected in the plants- not even in plants with mutated DCL1 (Reinhart et al., 2002). The lack of pre-miRNA in these *dcl1* plants (known as *caf-1* plants), together with the apparent nuclear localization of the DCL1 protein (Papp et al., 2002), suggests that DCL1 provides the Drosha functionality in plants, making the first cut that sets the register for miRNA maturation. Drosha does not contain helicase and PAZ domains found in DCR (Lee et al., 2003). DCL1 (or another enzyme yet to be identified)

then makes the second cut, which corresponds to metazoan Dicer cleavage, before the miRNA leaves the nucleus. A coupled second cut in the nucleus would explain why pre-miRNA-like RNAs do not accumulate to detectable levels in plants.

The mutations of Dicer-Like1 (*DCL1*) in *Arabidopsis* often exhibit different phenotypes by different investigators using different types of mutant screens. The situation is particularly complex when the mutations have pleiotropic effects. The RNase III gene with multidomains previously known as *EMBRYO DEFECTIVE76 (EMB76), SHORT INTEGUMENTS1 (SIN1), SUSPENSOR1 (SUS1)* and *CARPEL FACTORY (CAF)* was recently renamed as *DCL1* because the predicted protein sequence is homologous to the *Drosophila Dicer* and *C. elegans DCR-1*(Schauer et al., 2002). Bioinformatic approaches have identified three additional Dicer family members in Arabidopsis: *DCL2, DCL3* and *DCL4* (Schauer et al., 2002). The original *sus1* alleles are all presumed to be nulls, ranging from a >20 kb deletion to T-DNA disruption of essential protein domains (Errampalli et al., 1991). The *sins* (point) mutations lie within the RNA-helicase domain of DCL1, producing subtle changes in the predicted substrate-binding face (Ray et al., 1996). In contrast, the *caf-1* product presumably lacks a dsRNA-binding domain at the C-terminus (Jacobsen et al., 1999). Interestingly, one of the embryo-lethal alleles (*sus1-6*) lacks both the dsRNA-binding domains (McElver et al., 2001), suggesting that at least one functional dsRNA-binding domain is required for proper embryogenesis. Thus, the RNA-helicase domain and both dsRNA-binding domains are important for *DCL1* function *in vivo* (Schauer et al., 2002). After Dicer was found to be required for miRNA production in animal systems, *DCL1* of *Arabidopsis* was also identified to play a role in miRNA production. The accumulation of miRNAs is greatly reduced in *caf*, one of the d*cl1*

mutants in *Arabidopsis* (Reinhart et al., 2002; Park et al., 2002). This suggests that *DCL1* in *Arabidopsis* operates in miRNA metabolism as does Dicer in animals.

Surprisingly, *caf* mutants block neither PTGS nor siRNA production induced by self-complementary hairpin RNA (Finnegan et al., 2003). There are two explanations for this observation. First, *DCL1* may be only involved in the production of miRNAs, but not in siRNA processing. Second, *DCL1* may be involved in both pathways, but there is a redundancy for siRNA processing, because there are three additional Dicer homologues in *Arabidopsis*.

Following cleavage and nucleocytoplasmic export, the miRNA pathway of plants and animals appears to be biochemically indistinguishable from the central steps of PTGS pathways. MiRNAs were first reported to reside in the miRNA ribonucleoprotein complex (miRNP), which in humans includes the proteins elF2C2, the helicase Gemin3, and Gemin4 (Mourelatos et al., 2002). elF2C2 is a human Argonaute homolog and was later found to be a constituent of the human RISC which contains siRNAs as described in the section **1.1.2.2** (Martinez et al., 2002). Furthermore, the human miRNA *let-7* is associated with elF2C2 and is capable of specifying cleavage of an artificial target with perfect complementarity to the miRNA (Hutvagner and Zamore, 2002), although it remains possible that miRNP represents a particular subtype of RISC.

When the miRNA strand of the miRNA:miRNA* duplex is loaded into the RISC, the miRNA* appears to be peeled away and degraded. What is the mechanism for choosing which of the two strands enters the RISC? This largely lies in the relative base-pairing stability of the two ends of the duplex: for both of siRNA and miRNA duplexes,

the strand that enters the RISC is nearly always the one whose 5' end is less tightly paired (Khvorova et al., 2003; Schwarz et al., 2003). This observation suggests that a helicase-like enzyme (yet to be identified) samples the ends of the duplex multiple times, usually releasing the end before beginning to productively unwind the duplex; but it occasionally unwinds the duplex which has a less stably paired end, resulting in a strong bias for productive unwinding at the easier end (Khvorova et al., 2003; Schwarz et al., 2003). This elegant rule for predicting which strand of the duplex will enter the RISC was initially formulated based on observations and experiments in animal systems, but it also applies to plant siRNAs (Khvorova et al., 2003) and plant miRNAs. Its predictive value for the vast majority of plant and animal miRNAs strongly implies the existence of the miRNA:miRNA* duplex as a transient intermediate in the biogenesis of all miRNA, even those for which a miRNA* has not yet been cloned. Usually a miRNA:miRNA* duplex has a mismatch near the 5' end of miRNA. Meanwhile, siRNA duplexes can also be asymmetric, with only one of the two strands able to trigger RNAi. The siRNA strand of a duplex, whose 5' end has U:A pairing (more weak binding to the complementary strand), more readily incorporates into RISC, in contrast to, the another siRNA strand of the duplex whose 5' end has G:C pairing (Schwarz et al., 2003).

1.3.5 Mechanism for miRNAs to regulate their target mRNAs

The mechanism for *lin-4* and *let-7* to regulate their target mRNAs is well studied. The targets of these two miRMAs emerged from the analysis of genetic mutants that suppress the *lin-4* or *let-7* heterochronic mutant phenotypes (Lee et al., 1993; Slack et al., 2000; Wightman et al., 1993; Moss et al., 1997). The 3'UTR of the target mRNA bears more than one site of complementarity to the *Lin-4* and *Let-7* RNAs, each of which

contains bulge and loop mismatches. The significance of the miRNA complementary sites has been verified by mutations that render the site unresponsive to the regulatory RNA (Ha et al., 1996; Slack et al., 2000; Reinhart et al., 2000). These data indicate that *Let-7* and *Lin-4* RNAs down-regulate the expression of protein-coding mRNAs by base-pairing to partially complementary elements on those mRNAs. In the case of *Lin-4*, its target mRNAs are translationally repressed yet remain associated with polyribosomes, suggesting a block at a step after translational initiation (Olsen and Ambros, 1999).

However, it is not so simple. A let-7 miRNA acts like a siRNA by directing RNA cleavage in *Drosophila* and HeLa cells if it encounters a perfectly complementary target RNA (Hutvagner and Zamore, 2002). Moreover, irrespective of whether or not the target is perfectly complementary, the let-7 RNA occurs in a complex with RISC proteins (Mourelatos et al., 2002; Dostie et al., 2003). Thus, it seems that the same RISC is recruited by both siRNA and miRNA and that this complex mediates RNA cleavage if there is perfect complementarity with the target RNA, and translation arrest if there is a mismatch. Furthermore, *Arabidopsis miR171*, resembles an siRNA because it is perfectly complementary to its target mRNAs and directs specific cleavage of the predicted target RNA in inflorescences where miR171 was abundant (Llave et al., 2002). It is likely therefore that miR171 directs cleavage of a specific family of mRNAs in a tissue-specific manner.

When up to three mismatches are allowed in a computational search, putative mRNA targets have been identified for 14 of 16 miRNAs in *Arabidopsis*, as mentioned before. Now the question is whether these 1-3 nt mismatches between miRNAs and their targets allow miRNAs to direct the target mRNA cleavage. Ten miRNAs, which have 1-3

mismatches with their targets, can direct mRNA cleavage at the near central positions of the miRNA/mRNA complementary sites (Kasschau et al., 2003). The miR165/miR166-containing RISC complexes in wheat germ extracts functioned catalytically to cleave an imperfectly base-paired mRNA target from the PHAVOLUTA gene (Tang et al., 2003). My studies carried out independently during this period also support these results (see Section 5.3.3).

Plants with altered miRNA metabolism have pleiotropic developmental defects. Very recently direct evidence for miRNAs regulating specific aspects of plant morphogenesis has been demonstrated (Palatnik et al., 2003). In a genetic screen, the *JAW* locus was shown to produce a miRNA that can guide mRNA cleavage of several *TCP* genes that control leaf development.

The near-perfect complementarity approach failed to identify miRNA targets in *C. elegans* and *Drosophila* (Rhoades et al., 2002). However, predictions of more than 400 regulatory genes targeted by conserved mammalian miRNAs have been made by identifying mRNAs with conserved base-pairing to the 7 nt of 5' region of the miRNA (2-8) and evaluating the number and quality of these complementary sites. Thus, it appears that the plant miRNAs exhibit greater sequence identity with their targets than do the animal miRNAs. The *Let-7, Lin-4* and all predicted targets are in the 3'-UTR, whereas the majority of the plant miRNAs complementary sites were found to be within coding regions. But what mechanisms are adopted by those miRNAs that do not show near-perfect complementarities with mRNAs as suggested by the computational approach in plants? Obviously, without knowledge of the targets of all the animal and plant miRNAs, it is too early to speculate whether this reflects a true mechanistic difference between plant

and animal miRNAs. Indeed, recent studies have demonstrated that repressing translation of target mRNAs by miRNA is also found in plants. The miRNA172, which can base pair with the mRNA of a floral homeotic gene APETALA2, regulates APETALA2 expression primarily through translational inhibition (Chen X, 2003; Aukerman and Sakai, 2003).

Figure 1.2 shows the current model for the biogenesis and possible roles of miRNAs and siRNAs.

1.3.8 Viral suppressor and miRNA-controlled developmental pathway interaction.

After discovering miRNAs in plants, researchers started to investigate whether viral suppressors interfere with the miRNA regulation pathway, because PTGS and miRNA phenomena are overlapped. The first report of a suppressor interfering with miRNA pathway came from the work on HC-Pro from tobacco etch virus (Mallory et al., 2001). Before this study, it had been already proven that HC-Pro was able to block sense-transgene silencing by severely reducing or eliminating siRNA accumulation. This report further demonstrated that HC-Pro could suppress silencing induced by inverted-repeat transgenes, and viral amplicon-transcripts, and allowed accumulation of transcripts from the previously silenced loci also by blocking accumulation of the associated siRNAs. This suggests that HC-Pro may inhibit siRNA processing from double-stranded RNA precursors or destabilize siRNA. In contrast to siRNAs, the accumulation of endogenous miRNAs in HC-Pro transgenic plants was greatly enhanced. But as the targets of miRNAs in tobacco are unknown, there was no further investigation in the study.

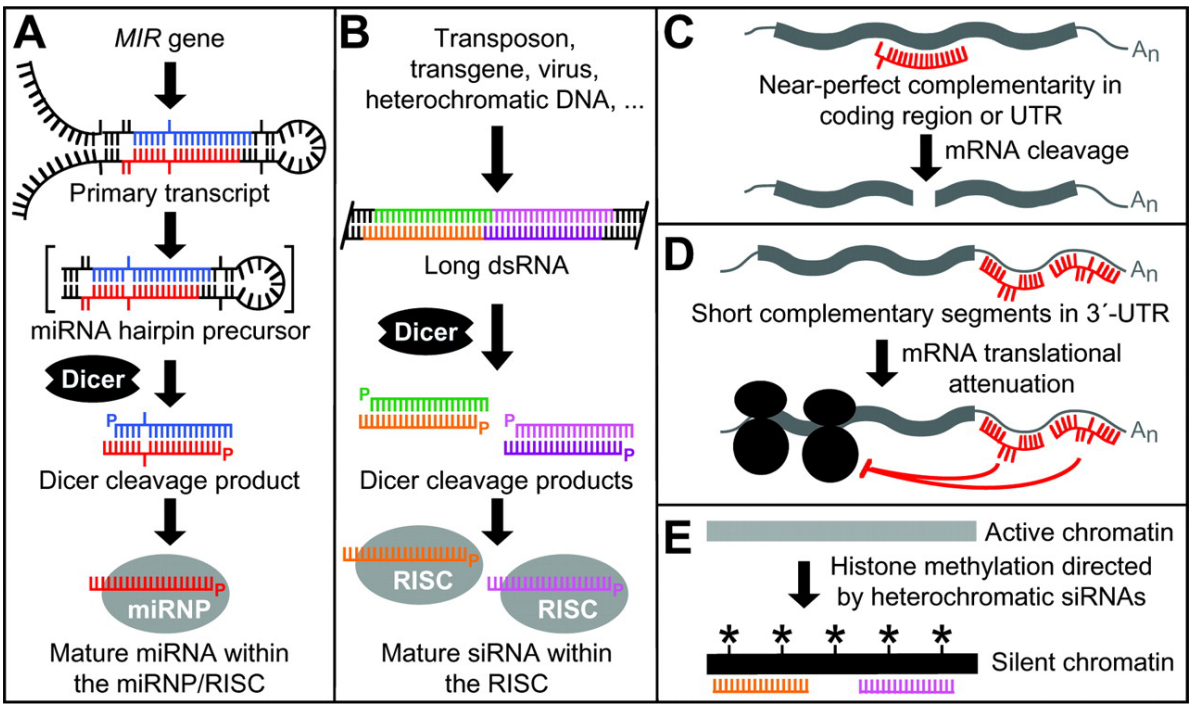


Figure 1.2 Current models for the biogenesis and possible roles of miRNAs and siRNAs.

(Adopted from Bonnie Bartel and David P. Bartel, 2003)

A, The portion of the primary transcript that contains the miRNA sequence (red) resides on one arm of a predicted stem-loop precursor structure. The transcription start and stop sites for miRNA primary transcripts have not yet been defined. In animals, the hairpin precursor (in brackets) is processed from the primary transcript, but such intermediates have not been detected in plants. Either the primary transcript or this processed hairpin is cleaved by Dicer to yield miRNA:miRNA* duplex (paired approximately 21-nt RNAs with 2-nt 3 overhangs, 5 phosphates, and 3 hydroxyls). One strand miRNA of this short-lived double-stranded intermediate accumulates as the mature miRNA (in red), which acts as a guide RNA within the miRNP/RISC complex. **B**, Long dsRNA is processed into many different siRNA species. siRNAs from both strands of the precursor accumulate within RISC complexes. **C**, The near perfect pairing between many plant miRNAs and their mRNA targets directs the RISC to cleave the target near the center of the complementarity site. This is also the classical mode of action for siRNAs during RNAi. **D**, Characterized animal miRNAs appear to recognize multiple sites in the 3-untranslated region (UTR) of target mRNAs. Because they bind to their targets with numerous mismatches, the miRNP/RISC does not cleave the message. Although the message levels remain constant, protein levels decrease, perhaps from translational attenuation. Whether any plant miRNAs act via this mechanism is not known. **E**, Some endogenous siRNAs, known as heterochromatic siRNAs, are thought to direct histone methylation, which is correlated with transcriptional silencing of the modified regions. Many of the non-miRNA small RNAs that have been cloned from Arabidopsis might act similarly.

The second report was done with a HC-Pro homolog encoded by Turnip mosaic virus (TuMV) in *Arabidopsis* (Kasschau et al., 2003). The HC-Pro transgenic and TuMV infected *Arabidopsis* plants caused abnormal development and sterility. The phenotypes were similar to *dcl1* mutants. Further study showed that HC-Pro suppresses miRNA-guided cleavage of target mRNAs, leading to elevated levels of target mRNAs, which may be responsible for the observed developmental abnormality and sterility. Thus, the basis for TuMV- and other virus-induced disease in plants may be explained, at least partly, by interference with miRNA-controlled development that may share components with the antiviral RNA-silencing pathway. Interestingly, miR162 can potentially target *DCL1* mRNA and a *DCL1*-derived RNA with the properties of a miR162-guided cleavage product was identified, suggesting that *DCL1* mRNA is subject to negative feedback regulation (Xie et al., 2003). In HC-Pro transgenic plants, *DCL1* mRNA accumulates to a higher level due to suppression of miR162-guided cleavage, and elevated levels of *DCL1* may drive miRNA precursor processing, leading to elevated levels of miRNAs (Xie et al., 2003).

Disease symptom caused by plant viruses is the sum of physiological and structural changes at the cellular level and alterations in physiology that are associated with the reduced growth and development of the whole plant. But up to the date, the basis of molecular mechanism of disease symptom is not clear. These recent finding that suppressors of PTGS interfere with miRNA regulation pathway suggests a novel molecular mechanism for the induction of plant viral diseases. The independent study described in this thesis supports this hypothesis.

1.4 TYMV

Turnip yellow mosaic virus (TYMV) is the type member of the tymovirus group of monopartite positive-strand RNA viruses (Markham and Smith, 1946). At least 20 tymoviruses are known (Matthews 1991).

TYMV virions are isometric and 28 nm in diameter. Their shells are regular icosahedra of 180 subunits of a single protein species which cluster in fives or sixes on the surface of the virion shell to form the 32 morphological subunits, which can be seen in negatively stained virions (Canady et al., 1995; 1996).

TYMV is prevalent both in cultivated and wild species of the *Brassicaceae* family of dicotyledonous plants. Tymovirus infection typically causes yellow mosaic symptoms. All plants infected with tymoviruses develop small characteristic vesicles on the outer membranes of their chloroplasts. These vesicles form as invaginations of outer chloroplast membranes, and the bilayers of these membranes and the vesicles are confluent (Matthews 1991).

1.4.1 Genomic organization of TYMV.

The TYMV genome is a 6813 nucleotides of ssRNA that is infectious when separated chemically from the virions (Morch et al., 1988b; Keese et al., 1989). The genomic organization is very compact. Only 192 of 6318 nt are noncoding. It encodes three open reading frames (ORF) (**Figure 1.3**). The largest ORF initiates at position 96 and ends at position 5630 to encode a protein of 206K with 1844 amino acids. The 206K protein is the only viral protein required for TYMV RNA replication in single cells

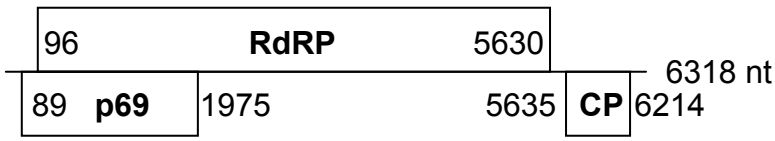


Figure 1.3 TYMV Genome Organization

(Weiland and Dreher 1989). It shows considerable amino acid sequence similarities with nonstructural putative replication proteins of several positive-strand RNA viruses and domains indicative of methyltransferase, NTPase/helicase, and RdRP activities have been highlighted in its sequence (Kamer and Argos, 1984; Gorbalenya et al., 1989; Rozanov et al., 1992). The 206K protein also contains a papain-like cysteine proteinase domain located between the methyltransferase and the NTPase/helicase domains (Bransom et al., 1994; Rozanov et al., 1995) that is responsible for the cotranslational proteolytic cleavage of the 206K protein in vitro (Bransom et al., 1996). Mapping of the cleavage site (between A1259 and T1260) revealed that the resulting N-terminal protein product of 141 kDa (141K) contains the methyltrasferase, proteinase, and NTPase/helicase motifs, whereas the C-terminal protein 66K protein encompasses the RdRP domain (Kadare et al., 1995; Bransom et al., 1996). This cleavage was also demonstrated to be functional in vivo (Prodhomme et al., 2001), and both the 150K and 70k viral proteins appear to be essential for the replication of TYMV RNA genome (Weiland and Dreher 1993). The second ORF encodes the 20K coat protein (nt from 5635 to 6214). The coat protein is translated from a subgenomic RNA with 794 nt. The third ORF starts from 89 and terminates at 1975 to encode a protein of 69K with 629 amino acids, which is called p69 (Skotnicki et al., 1992b; Bozarth et al., 1992) (**Figure 1.3**). There is a 105-nt 3' untranslated region, which contains a tRNA-like structure (Skuzeski et al., 1996).

1.4.2 p69 Protein of TYMV

The first publication on the study of TYMV p69 appeared in 1992 (Bozarth et al., 1992). A p69 specific antiserum was produced using a synthetic peptide corresponding to

the C-terminal 14 amino acids of p69 and p69 was detected both *in vitro* and *in vivo*, but it migrated slower in SDS-PAGE as 75 kDa, perhaps due to its high pI of 12.1. Although p69 was abundant in the systemically infected leaves in Chinese cabbage and *Arabidopsis* plants, it was not detectable in inoculated leaves. TYMV mutant RNAs with stop codons inserted at nucleotides 139, 178, and 178/224 in p69, which had no effect on coding potential of the overlapping replicase, replicated to wild-type levels in turnip or Chinese cabbage protoplasts, but were not detectable in either inoculated leaves or systemic leaves. The results suggest that p69 is dispensable for replication, but is required for viral spread.

Further study by the same laboratory showed that a spontaneous TYMV mutant produced more severe, almost uniformly chlorotic symptoms in the systemically infected leaves of Chinese cabbage plants (Tsai and Dreher, 1993). The severe symptoms accompanied increased yields of virus in infected tissue. The increased symptom severity is due to the single nucleotide substitution U1888→C, which results in a tyrosine to histidine substitution in the p69 protein, but not in the coding by the overlapping replicase. The mutation results in a fourfold higher accumulation of viral products in systemically infected Chinese cabbage leaves, but does not affect viral replication in isolated protoplasts. These results not only further confirm that p69 is dispensable for RNA replication on single cells and is required for virus spread in the plant, but also imply that p69 is a virulence determinant.

A recent study has demonstrated that p69 protein is selectively degraded through a ubiqitin-dependent 26S proteasome degradation pathway, by using *in* vitro rabbit reticulocyte lysate system (Drugeon and Jupin, 2002), which suggests that the transient

nature of accumulation of p69 protein in the course of infection may be due to this selective proteolysis.

1.5 Rationale and Aims of the project.

In plants, PTGS is an adaptive immune system targeted against viruses. It has been greatly used to develop crops with viral disease resistance, which is easily obtained by transforming viral genes into plant genomes (Baulcombe, 1996; Wang and Waterhouse, 2002). As a counter-strategy, however, these pathogens have evolved suppressors of the silencing antiviral response, a necessary adaptation for successful infection of their hosts. This strategy may not work as effectively as it should when transgenic plants are infected with a heterologous virus that carries a strong suppressor of PTGS (Mitter et al., 2003). Viral suppressors of RNA silencing are diverse. Suppressors encoded by different virus families share no obvious common structural or sequence motif and, based on suppression of transgene silencing, they appear to act against different stages of the PTGS mechanism (Brigneti et al., 1998; Kasschau and Carrington 1998; Voinnet et al., 1999; Li and Ding, 2001; Guo et al., 2002). Strikingly, RNAi suppressors were also found in animal viruses by using *Drosophila* cell line system (Li et al., 2002; Li et al., 2004).

Almost all assays of suppression of PTGS were performed in *Nicotiana* spp such as *N. tabacum* and *N. benthamiana* in the previous studies due to some obvious advantages of tobacco plants. In *N. benthamiana*, PTGS can be efficiently induced by agro-infiltration. In addition, grafting is easily carried out with these plants to test the systemic signaling of PTGS. However, a molecular understanding of the function and

mechanism of viral suppression of PTGS requires the availability of the host genome sequence, which is not available for tobacco.

In this thesis, I describe an *Arabidopsis* system to examine the function and mechamism of viral suppression of PTGS. A number of distinct PTGS triggers are active in *Arabidopsis* as in other species and include transgenes that express abundant sense-RNAs, inverted-repeat RNAs and replication-competent viral amplicons. In contrast to *Nicotiana* spp, however, several genetic mutants defective for PTGS are well-characterized in *Arabidopsis*. Most importantly, the genome sequence of *Arabidopsis* is available, over 100 miRNAs have been sequenced and many of their target mRNAs either predicted or verified. Therefore, the aims of thesis were:

1. To determine if TYMV, which is highly pathogenic to *Arabidopsis*, encodes a PTGS suppressor.

2. To analyze the mechanism of PTGS suppression by the identified suppressor.

3. To investigate if the TYMV suppressor inferferes with the miRNA pathway and plays a role in viral pathogenesis.

Chapter 2

General materials and methods

2.1 Plant materials and growth conditions

Arabidopsis lines used in this study were C24, Col-0, Ws-0, L1 (GUS silencing line) (Mourrain et al., 2000d), Amp (PVX-GFP silencing line) (Dalmay et al., 2000a), GFP (35S-*GFP* expression line) (Dalmay et al., 2000a), GxA (silencing line) (Dalmay et al., 2000a), Amp-TYMV (transgene expression line), IR*Rbx1* (inverted-repeated Rbx1 silencing line) (Xu et al., 2002c), IR*PDS* (inverted-repeated *PDS* silencing line), IR*GFP* (inverted-repeated GFP silencing line). *Aradidopsis* mutants used in the study were *sde1* (a RdRP loss of function mutant in Amp x GFP genetic back ground) (Dalmay et al., 2000b), *sgs2* (another RdRP loss of function mutant in L1 genetic background) (Mourrain et al., 2000). Transgenic *Nicotiana benthamiana* carrying the GFP ORF was line 16C, which carries one copy of GFP-transgene at a single locus in homozygous condition (Ruis et al., 1998).

For plants grown in soil, *Arabidopsis* seeds were allowed to imbibe on water-wetted filter paper at 4^{o}C for 7d, and then planted on Arabidopsis mix (three parts Florobella potting compost/1 part sand). For plants grown on medium, seeds were surface sterilized and sown on MS medium or MS medium supplemented (where appropriate) with chemicals or antibiotics. The seeds were then chilled in a cold room at 4^{o}C for 7 d. In both cases, the plants were then grown in a growth room (16 h light/8 h darkness photoperiod, 20-23^{o}C).

2.2 Chemical solutions and growth media

TAE (Electrophoresis running buffer): 40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.2.

TBE (Electrophoresis running buffer): 89 mM Tris-borate, 2 mM EDTA, pH 8.3

TE: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

LB medium: 1% (w/v) Bacto-tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0.

Denhardt's III (100x): 2% BSA, 2% Ficoll 400, 2% Polyvinyl-pyrrolidone 360, 10% SDS.

20xSSC: 3M NaCl, 0.3M Trisodium citrate.

Hybridization Buffer: 0.35 M Na_2HPO_4, 0.15 M NaH_2PO_4, 7% SDS, 1 mM EDTA.

Murashige and Skoog (MS) medium: 16 mg/L $MnSO_4 \cdot H_2O$, 8.6 mg/L $ZnSO_4 \cdot 7H_2O$, 6.2 mg/L H_3BO_3, 0.83 mg/L KI, 0.25 mg/L Na_2MoO_4, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 0.025 mg/L $CoCl_2 \cdot 6H_2O$

2.3 Cloning procedure

2.3.1 Polymerase chain reaction (PCR)

Reaction was set up to 50 ul total volume in a 0.25 ml microfuge tube as follows: 1 x reaction buffer, 0.2 mM of each dNTP, 0.4 uM of each primer, 0.1 ug template DNA and 1.25 Units of Taq or Pfu DNA polymerase. Amplification was carried out in a Programmable Thermal Controller (Model PTC-100, *MJ Research*) using the following program: 94^{o}C for 5 minutes; followed by 94^{o}C for 45 seconds, 55^{o}C for 45 seconds, 72^{o}C for various times (depends on the length of amplified DNA fragments) for 30 cycles; 72^{o}C for 10 minutes. PCR products were separated by electrophoresis on 1.0% agarose gels and purified as described in section 2.3.3.

2.3.2 Reverse Transcription-PCR (RT-PCR)

The Titan® One Tube RT-PCR Kit from Roche molecular Biochemicals Company was used. Total 50 ul reaction volume was set up as follows: 0.2 mM of each dNTP, 0.4 uM of each primer, 1 ug total RNA, 5 mM DTT, 5 U RNase Inhibitor, 1 x reaction buffer and 1 ul Enzyme mix. The following program was used 50^{o}C for 30 minutes; 94^{o}C for 5 minutes; followed by 94^{o}C for 45 seconds, 55^{o}C for 45 seconds, 68^{o}C for 4 minutes for 35 cycles; 68^{o}C for 7 minutes. The amplified fragments were purified as PCR products.

2.3.3 Purification of DNA from agarose gels

DNA bands were excised from the agarose gel under long wavelength UV light and the DNA fragments were purified through Qiagen Gel Extraction Kit as described by the manufacturer.

2.3.4 Isolation of plasmid DNA from *E. coli*

Frozen glycerol stock of bacteria was streaked on LB plate with appropriate antibiotics (ampicillin 100 ug/ml or kanamicin 50 ug/ml), and grown overnight at 37^{o}C. Liquid cultures were set up by inoculating single colony to LB medium containing appropriate antibiotic and incubating at 37^{o}C with vigorous shaking. Minipreparation of plasmid DNA was carried out using the Qiagen kits following the protocols provided by the manufacturer.

2.3.5 Preparation of plasmid vectors for cloning

2.3.5.1 End-blunting of digested plasmid DNA with Large (Klenow) Fragment

End-blunting of restricted plasmid DNA for cloning was performed using large klenow fragment. Klenow (1 U/ug DNA) and dNTP (a final concentration of 33 uM of each dNTP) were directly added to the reaction mix in which plasmid DNA was digested

with restriction endonuclease (NEB). The reaction was incubated at 25°C for 15 minutes, then subjected to agarose gel electrophoresis and DNA purification.

2.3.5.2 Dephosphorylation of restricted plasmid DNA

The restricted plasmid DNA for cloning was dephosphorylated using calf intestinal alkaline phosphatase (CIP, NEB). Digested DNA was mixed with CIP (0.5 U/ug vector DNA) and incubated at 37°C for 1 hour in the presence of appropriate 1x NEBuffer. The dephosphorylated DNA was purified using Qiagen gel extraction kit directly as described in the QIAquick Spin Handbook.

2.3.6 Ligation of DNA inserts into plasmid vectors

Ten ng of linearised vector DNA was mixed with the insert DNA at a molar ratio of 1:3 (vector : insert), or 50 ng pGEM®-T Easy vector was mixed with the PCR or RT-PCR products without any treatment at a molar ratio of 1:3. The ligation was carried out with Rapid DNA Ligation kit (Roche) at room temperature for at least 5 minutes.

2.3.7 Transformation of bacterial with plasmids

2.3.7.1 Preparation of *E. coli* competent cells for heat-shock transformation

Competent cells of *E. coli* DH5α were prepared as described by Inoue et al. (1990). Single colony of DH5α from a fresh streaked plate was inoculated to 250 ml of SOB medium (2% Bacto tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$) in a 1-liter flask, and grown to an OD_{600} of 0.6 at 18°C, with vigorous shaking (200-250 rpm). The flask was removed from incubator and placed on ice for 10 minutes, then the culture was centrifuged at 2500xg for 10 minutes at 4°C. The pellet was resuspended in 80 ml of ice-cold TB (10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl). All components except for $MnCl_2$ were mixed and the pH was adjusted to 6.7

with KOH. Then, $MnCl_2$ (filter-sterilized and store at 4°C) was added in and incubated on ice for 10 minutes, and spun down as above. The cell pellet was gently resuspended in 20 ml of TB, and DMSO was added with gentle swirling to a final concentration of 7%. After incubation on ice for 10 minutes, the cell suspension was dispensed into 1.5 ml microfuge tubes and immediately immersed in liquid nitrogen. The frozen competent cells were stored at -80°C.

2.3.7.2 Preparation of electro-competent Agrobacterium

Single colony of EHA105 strain was inoculated to 5 ml LB medium containing 50 ug/ml of kanamycin and 50 ug/ml rifampicin, and incubated at 28°C. The overnight culture was diluted in 100 ml LB medium to an OD_{600} of 0.04 to 0.08 and incubated at 28°C for approximately 4 hours to an OD_{600} of 0.5. The bacterial were spun down at 10,000 xg for 10 minutes at 4°C. The pellet of bacterial was washed first with 40 ml of 1 mM HEPES (pH 7.0), then 40 ml of 1 mM HEPES (pH 7.0), 10% glycerol. The suspended cells were dispensed by 100 ul into microfuge tubes and immediately immersed in liquid nitrogen. The frozen competent cells were stored at -80°C.

2.3.7.3 Transformation of E. coli cells using heat-shock method

E. *coli* DH5α competent cells (200 ul) were mixed with 10 ul of ligation reaction mixtures, kept on ice for 30 minutes and subjected a heat shock at 42°C for 1 minute. After chilling on ice for 1 minute, the mixture was mixed with 800 ul of LB medium containing no antibiotics and incubated at 37°C for 1 hour. Two hundred ul of bacterial culture was plated with appropriate antibiotics and incubated at 37°C overnight.

2.3.7.4 Transformation of Agrobacterium cells by electroporation method.

Miniprep plasmid DNA (~0.1 ug) was added to 50 ul of frozen competent cells and incubated on ice for 1-2 minutes. The mixture was placed in an ice-cold 0.2 cm

electroporation cuvette and electroporation was performed using a BioRad Gene Pulser. The parameters were set as 25uF, 400 Ω for a 2.5 KV pulse, followed with an 8-9 ms delay. Then 1 ml of LB was immediately added to the electroporation solution, then transferred to a sterile culture tube and incubated at 28^{o}C for 2 hours to allow cell recovery and antibiotic resistance gene expression. Twenty ul of the bacterial culture was spread on a LB plate containing appropriate antibiotics (Kanamycin 50 ug/ul, Rifampicin 10 ug/ml) and grown at 28^{o}C for 2 days.

2.4 DNA sequencing

DNA sequencing was performed using the ABI PRISM dRhodamine Terminators Cycle Sequencing Ready Reaction Kit as described in manufacturer's protocol.

2.5 Transformation of *Arabidopsis* by *Agrobacterium* vacuum-infiltration transformation method

Primary inflorescences of *Arabidopsis* plants were clipped at their bases and secondary inflorescences were allowed to grow until they started to show open flowers.

Agrobacterium EHA105 cells carrying appropriate Ti plasmid were grown in LB medium with antibiotics until OD_{600} reached 2. The bacteria were spun down at 4,000 xg for 10 minutes. The pellet was resuspended in ½ MS liquid medium with 5% sucrose, 44 nM 6-benzyl aminopurine and 0.005% silwet L-77 (pH 5.7). The final bacterial suspension had an OD_{600} of 0.8. The plants were submerged in the Agrobacterial suspension and vacuum was drawn until the solution bubbled vigorously, when the vacuum was quickly released. Plants were covered with a plastic dome overnight, and then cultivated in the growth room until seeds matured and dried (Clough and Bent, 1998).

2.6 In vitro transcription with RNA polymerases

2.6.1 Preparation of radioactive RNA probes

The reaction mix for making radioactive RNA probe was made as follows: 0.1 M DTT, 2 ul; 3.3 mm ATP/CTP/GTP, 3 ul; 0.3 mM UTP, 0.8 ul; RNase inhibitor (40 units/ul, *Promega*), 0.5 ul; 10 x Transcription Bufer, 2 ul; linearised template DNA (0.5 ug/ul), 2 ul; α-^{32}P UTP (10 mCi/ml), 4 ul; RNA polymerase (either T7 or T3, SP6 from *NEB*), 2 ul; H_2O, 3.7 ul to a total volume of 20 ul. The reaction mix was incubated at 37^{o}C for 1 hour, followed by treatment of DNase I (RNase free, Roche) for another 15 minutes to remove template DNA. The labeled probe was ready for use.

2.6.2 Preparation of infectious transcripts

In vitro transcription of capped mRNA was performed using the T7 Cap-Scribe Kit (Boehringer Mannheim). The following items were mixed on ice and made up to a final volume of 20 ul [4 ul Cap-Scribe buffer (5x); 1 ul linearized template DNA (0.5 ug/ul); 13 ul RNase-free H_2O; 2 ul T7 RNA polymerase (20 units/ul)]. The reaction mix was incubated at 37^{o}C for 1 hour, when an aliquot of 0.5 ul was removed and analyzed by agarose gel electrophoresis to estimate the size and amount of transcripts. The remainder was stored at -20^{o}C until use.

2.7 Plant inoculation

Arabidopsis plants with 6 leaves at approximately 4 weeks old were used. The young fully expanded leaves of the plants were dusted with carborundum and inoculated with the different inocula diluted in 10 mM sodium phosphate: capped RNA transcripts (~5 ug/plant), or sap from infected plants (100 x dilution).

2.8 Total RNA extraction from plants

Total RNAs were isolated from plants as described by Verwoerd et al. (1989). Briefly, leaves (0.1-1 g) were grounded into powder in liquid nitrogen. The powder was transferred to a 2 ml microfuge tube containing 1 ml hot phenol buffer (Napoli et al., 1990) followed by vortexing for 20 seconds. Chloroform (500 ul) was added and vortexed for 20 seconds. After centrifugation at 10,000 xg for 10 minutes at room temperature, the aqueous phase was removed and mixed well with an equal volume of 4M LiCl and left at -20^{o}C for overnight. The RNA pellets were obtained by centrifugation at 10,000 xg for 15 minutes at 4^{o}C, and dissolved in 300 ul DEPC-treated TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH8.0). About 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol were added to the dissolved RNA. The mixture was left at -20^{o}C for at least 20 minutes. The RNA was precipitated by centrifugation at 10,000 xg for 15 minutes at 4^{o}C. The pellet was washed with 70% ethanol, dried in a SpeedVac and resuspended in 50 to 100 ul DEPC-treated H_2O or TE buffer, and stored at -20^{o}C.

2.9 Extraction of plant DNA

Plant DNA was isolated by ethanol-precipitation of the LiCl-supernatant in the procedure of total RNA extraction. The DNA pellet was washed with 70% ethanol and resuspended in sterile water.

2.10 Random labeling of DNA with ^{32}P dCTP

Redioactive Random Labeling kit (Amersham) was used. Twenty-five ng DNA fragments of desirable genes (either PCR products or products from restriction enzyme digestion) in 45 ul TE buffer were boiled for 5 minutes, and then chilled on ice for 5

minutes. The denatured DNA solution was added to labeling mixture and 5 ul of [α-^{32}P] dCTP was also added. The mixture was incubated at 37^{o}C for 10 minutes and then boiled for 5 minutes, followed by cooling on ice prior to use.

2.11 End labeling of DNA with r-^{32}P ATP

Polynucleotide kinase was used for labeling of 5'-hydroxyl ends of DNA fragments. The reaction was set up as follows: 20 pM DNA (5'-OH), 1x Buffer, 20 pM [r-^{32}P] ATP, 10 units polynucleotide kinase (Roche), water to 20 ul. The mixture was incubated at 37^{o}C for 30 minutes and stopped by cooling on ice. The probe was ready for use.

2.12 Northern blot hybridization

2.12.1 Preparation of formaldehyde-denaturing RNA gel

For a 200 ml (15x20 gel casting tray) 1.2% agarose gel, 2.4 g of agarose in 174 ml RNase-free H_2O was boiled in a microwave oven until completely dissolved. When the mixture had cooled to 60^{o}C, 20 ml of 10x MOPS buffer [0.5 M MOPS, 0.01 M EDTA, pH 7.0] and 6 ml of 37% formaldehyde were gently mixed in thoroughly, and then poured into a RNase-free gel tray.

2.12.2 Sample preparation and electrophoresis

Appropriate amount of total RNA was aliquoted to 1.5 ml microfuge tube and dried in a SpeedVac. The RNA pellet was dissolved in 10 ul of sample buffer [10X MOPS buffer/formamide/formaldehyde/H_2O (1:1.8:5:2.2)], heated at 65^{o}C for 10 minutes, and cooled on ice. After 2 ul of 6X RNA loading buffer were added, the RNA samples were

loaded on the formaldehyde denaturing RNA gel. The gel was run at 100 V for 2-3 hours until the bromophenol blue reached the bottom of the gel.

2.12.3 RNA transfer from gel to nylon membrane

The gel was rinsed in 5X SSC (20x SSC: 3 M NaCl, 0.3 M trisodium citrate) for 10 minutes. RNA transfer from gel to a Hybond-N nylon membrane was performed with a TransVac Vacuum Blotting Unit (TE80, Hoefer). After transfer, the membrane was briefly washed in 5x SSC, and fixed in a GS Gene Linker UV Chamber (Sigma, 150 mj). To certify equal loading of RNA samples, the membrane was stained with methylene blue stain solution (0.04% in 0.5 M sodium acetate, pH 5.2) to estimate the amount of RNA by visualization.

2.12.4 Hybridization

Prehybridization solution (10 ml) was made with 0.35 M Na_2HPO_4, 0.15 M NaH_2PO_4, 7% SDS, 1 mM EDTA, finally added with 100 ul of 10 mg/ml denatured salmon sperm DNA. Prehybridization was performed at 65^{o}C for at least 1 hour in a hybridization shaker. ^{32}P-labeled DNA or RNA probe was directly added into pre-hybridization mix, and incubated at 65^{o}C for more than 8 hours. The membrane was washed once in 2X SSC/0.1% SDS at 65^{o}C for 30 minutes, twice in 0.2X SSC/0.1% SDS for 20 minutes, and then exposed to X-ray film for autoradiograghy.

2.12.5 Stripping probes from membranes

For reprobing the membrane, the probe was stripped with boiled stripping solution [0.1% SDS, 10 mM Tris (pH7.4)] followed by incubating at 65^{o}C for 30 minutes with gentle shaking.

2.13 Southern blot analysis

2.13.1 DNA gel electrophoresis

DNA (10-20 ug) was digested with 30-50 units of an appropriate restriction enzyme for at least 6 hours, followed by ethanol precipitation. The digested DNA was dissolved in 20 ul TE buffer with 4 ul of 10x DNA loading buffer and then loaded onto a 1.2 or 1.5% agarose gel containing 0.5 ug/ml ethidium bromide. The gel was run at 20 V for 12-16 hours until the bromophenol blue reached 2/3 of the way of the bottom of the gel. The DNA was visualized on a UV transilluminator and photographed to estimate the amounts of DNA loaded and the quality of DNA digestion.

2.13.2 Transfer of DNA from gel to membrane

The gel was treated with 500 ml of 0.2 M HCl for 10-20 minutes until the bromophenol blue turned yellow, washed briefly in water, and then treated with 500 ml of 0.4 M NaOH for 30 minutes. When the bromophenol blue turned blue again, the gel was put in 500 ml of neutralization buffer [1.5M NaCl, 0.5 M Tris-HCl, 0.001 M EDTA, pH 7.2] for 30 minutes. The DNA transfer from the gel to Hybond-N nylon membrane was carried out as described in section 2.11.3. The membrane was rinsed briefly in 5X SSC and then UV crosslinked.

2.13.3 Hybridization

Hybridization was performed as described in section 2.11.4.

2.13.4 Stripping probes

The membrane after hybridization was put in 0.2 M NaOH for 10 minutes, 0.2 M Tris-HCl (pH7.4), 0.1% SDS for 10 minutes; 0.1x SSC, 0.1% SDS for 10 minutes. The stripped membrane was wrapped with filter paper and kept at 4^{o}C.

2.14 Agro-infiltration

Induction of transgene-GFP PTGS was carried out by agro-infiltration according to the protocol used in Baulcombe's lab (Voinnet et al., 2000). The details are as follows:

The *Agrobacterium* strain EHA105 was first streaked on LB medium plate containing the selection antibiotics (kanamycin 50 ug/ml, rifampicin 10 ug/ml) and incubated at 28^{o}C. A single colony was picked into a 2 ml LB medium with above antibiotics, and grown at 28^{o}C for 48 hours with vigorous shaking. One ml of the culture was transferred to 50 ml LB medium with the antibiotics and 10 mM MES (pH5.6) and 20 ul of 100 mM acetosyringone. After incubation at 28^{o}C for 16 hours with vigorous shaking, OD_{600} of the culture reached 1.0. The bacteria were spun down at 4000 xg for 10 minutes, the pellet was resuspended in 50 ml 10 mM $MgCl_2$, and then 75 ul of 100 mM acetosyringone was added. The bacteria were kept at room temperature for at least 3 hours without shaking.

Infiltration was performed with a 1ml syringe without needle. *Nicotiana benthamiana* plants with 5-7 leaves were used for infiltration. Two well-expanded leaves were punched with a needle, the syringe was applied to the hole on the leaf with blocking by finger from the other side. The syringe barrel was gently pushed and the bacteria suspension was delivered into the intercellular space of the leaf.

2.15 GFP imaging

GFP fluorescence in whole plants was monitored by using a high-intensity ultraviolet lamp (Model SB-100P/F, 365 nm, Spectroline). Photographs were taken with a Kodak Ektachrome 400 color reversal film through a Wratten 8 filter (Kodak). Exposure time varied up to 70 seconds depending on the intensity of the fluorescence and the distance of the camera and lamp from the plants.

2.16 GUS staining

Plants or plant tissues were put into GUS staining solution [10 mM EDTA, 0.1% Triton X-100, 2 mM $Fe^{2+}CN$, 1 mg/ml X-gluc in 50 mM phosphate buffer (pH 7.0)] and degassed under vacuum for 15 min. and then incubated at room temperature until a signal appeared. After staining, 70% ethanol was used to remove chlorophyll for tissue localization.

2.17 Isolation of low molecular weight (LMW) RNA from plants

The method for isolation of LMW RNA from plants is based on that for plant DNA extraction with slight modifications (Guo and Ding, 2002). The aqueous phase obtained after phenol/chloroform extraction was first mixed with 1/10 volume of 3 M sodium acetate and 2.5 volumes of 100% ethanol, and kept at -20^{o}C for at least 2 hours. The mixture was centrifuged at 10,000 xg for 20 minutes at 4^{o}C. The pellet obtained was air-dried and resuspended in 1x TE buffer (pH 8.0) following by addition of equal volume of 4 M LiCl dissolved in DEPC-treated water. After leaving at -20^{o}C for 2 hours, the mixture was spun at 10,000 xg for 20 minutes at 4^{o}C. The supernatant containing DNA and LMW RNA was collected, while the pellet contained high molecular weight (HMW) RNA. Three volumes of 100% ethanol were added to the supernatant and the mixture was incubated at -20^{o}C for 2 hours. LMW RNA and DNA were co-precipitated by centrifugation as above, washed with 70% ethanol, air-dried and resuspended in DEPC-treated H_2O. This extract of LMW RNA and DNA can also be used for Southern blotting.

2.18 Detection of siRNA and miRNA

2.18.1 Detection of siRNA

2.18.1.1 Electrophoresis of LMW RNA on a sequencing gel

LMW RNA was separated on a sequencing gel [16% polyacrylamide (19:1), 7 M Urea, 0.5X TBE]. Before loading, 20-40 ug of LMW RNA extracts in a volume of 20 ul were mixed with one volume of formamide, boiled for 5 minutes, quickly chilled on ice, and then 6X loading buffer was added. DNA oligonucleotides were used as molecular weight markers. The gel was run in 0.5X TBE buffer at 300V until bromophenol blue reached the bottom of the gel, then rinsed in 0.5X TBE buffer. LMW RNA was transferred from gel to a Hybond-N nylon membrane in a 1X TBE buffer at 3mA/cm2 for 30 minutes with a Semi-Dry Transfer Cell (BIO-RAD). The membrane was equilibrated on several layers of filter paper soaked in 20x SSC for 30 minutes, then fixed by UV cross-linking with a Gene Linker. The fixed membrane can be stained by methylene blue to monitor equal RNA loading.

2.18.1.2 Preparation of ^{32}P-labeled RNA probe for LMW RNA

^{32}P-labeled RNA probes transcribed *in vitro* from plasmid templates were prepared as described in Section 2.5.1. Ten units of RNase-free DNase I were added to the reaction mix (20 ul) of *in vitro* transcription to remove the DNA templates at 37^{o}C for 15 minutes. In order to reduce probe sizes into about 50 nt in average size, 300 ul of carbonate buffer [80 mM $NaHCO_3$, 120 mM Na_2CO_3] was added to the 20 ul transcription solution and incubated at 60^{o}C for about 3 hours. After that, 20 ul of 3M sodium acetate was added to the hydrolyzed RNA probes and the probes were ready for use.

2.18.1.3 Hybridization

Pre-hybridization of membrane was carried out in hybridization solution [50% formamide; 7% SDS; 50 mM $NaPO_4$ (pH 7.0); 0.3 M NaCl; 5X Denhardt's solution; 100 ug/ml of sheared, denatured salmon sperm DNA] at 40^{o}C for at least half an hour. After addition of the labeled probe, hybridization was performed at 40^{o}C overnight. The membrane was washed twice with 2X SSC/0.2% SDS at 50^{o}C, once with 0.2X SSC/0.1% SDS, and then exposed to X-ray film. For re-probing the membrane, the probe was stripped with stripping solution (0.2% SDS, 10 mM Tris-HCl, pH 7.5) at 90^{o}C for 1 minute.

2.18.2 Detection of miRNA

2.18.2.1 Electrophoresis of siRNA on a sequencing gel

50 ug of total RNAs were used. All procedures to detect miRNA were the same as those described in siRNA detection.

2.18.2.2 Preparation of probes and hybridization

5'-hydroxyl ends of 21-22 mer oligo-DNAs were labeled with [r^{32}P]-ATP by direct phosphorylation as described in Section 2.10. miRNA hybridization was the same as described in Section 2.17.1.3.

2.19 Real-time PCR

2.19.1 Reverse transcription

Total RNA was extracted from whole plants as used in the miRNA assay. For synthesis of complementary DNA, SuperScrip^{TM}II RNase H^{-} Reverse Transcriptase (Invitrogen) was used. A 20-ul reaction volume was carried out as follows:

The first step:1 ul Oligo $(dT)_{15}$ (500 ug/ml), 5 ug total RNA, 1 ul 10 mM of each dNTP, sterile, distilled water to 12 ul was mixed; the mixture was heated 65^{o}C for 5 min and quickly chilled on ice.

The second step: the mixture was added 4 ul 5X First-strand buffer, 2 ul 0.1 M DTT, 1 ul RNaseOUT^{TM} recombinant Ribonuclease Inhibitor (40 U/ul). The contents were mixed gently and incubated at 42^{o}C for 2 min.

The third step: the above mixture was added 1 ul (200 U) of SuperScript^{TM}II^{aa} and incubated 50 min at 42^{o}C. Finally, the cDNA was ready for use.

2.19.2 Real-Time PCR

The cDNA solution from the reverse transcription (senction 2.19.1) 0.5ul was used as template for PCR. The number of cycles was determined by LightCycler Instrument-Real time PCR machine (Roche Applied science). The LightCycler-FastStartDNA Master SYBR Green Kit was used in the PCR. All procedures followed the protocol given by the manufacturer. When PCR was in the log-linear phase before the later plateau phase, the reactions were stopped.

2.19.3 Hybridization

PCR products were blotted to a nylon membrane and hybridized with probes derived from corresponding genes, respectively. These were performed as described in the section of Southern blot analysis.

2.20 Purification of mRNA from total RNA

Oligotex® mRNA Midi kit (Qiagen) was used to purify mRNA from total RNA. The protocol was given by the manufacturer. Briefly, 500 ug total RNA was mixed with 45 ul Oligotex. Finally 50 ul hot (70^{o}C) Buffer OEB was used to elute the mRNA.

2.21 RNA ligase-mediated rapid amplification of cDNA ends

The FirstChoiceTM RLM-RACE kit (Ambion The RNA Company) was used.

1: 250 ng mRNA was ligated to the 5' RACE Adapter. The reaction was set up as follows: 250 ng mRNA, 1 ul 5' adaptor, 1 ul 10X buffer, 2 ul T4 RNA ligase (2.5 u/ul), nuclease-free water to 10 ul and incubated at 37^{o}C for 1 hour.

2: Reverse transcription was performed as follow: 2 ul ligated RNA, 4 ul dNTP mix, 2 ul random Decamers, 2 ul 10X RT buffers, 1 ul RNase Inhibitor, 1 ul M-MLV Reverse Transcriptase, nuclease-free water to 20 ul. The mix was incubated at 42^{o}C for 1 hour.

3: Two ul synthesized cDNA was used to carry out the first round of PCR as described in the section 2.3.1. The 5' primer was 5'RACE outer primer. The 3' primers were gene specific outer primers.

4: Two ul of PCR products from last round PCR were used for nested PCR. The 5' primer was 5'RACE inner primer. The 3' primers were gene specific inner primers.

5: In each case, a unique gene-specific DNA fragment was amplified. The PCR products were gel purified as described in Section 2.3.3 and cloned into pGEM-T Easy vector (Promega) as described in Sections 2.3.5, 2.3.6 and 2.3.7.

6: Plasmid DNAs were extracted from single colonies as described in the section 2.3.4.

7: M13 Forward and Reverse primers were used to sequence individual clones.

Note: Methods used in this thesis referred to standard procedure (Sambrook et al., 1989) or manufacturer's protocols except where indicated.

Chapter 3
TYMV suppresses PTGS in *Arabidopsis*

3.1 Introduction

Virus-host interactions are governed by complex sets of viral and host genes that specify their compatibility. Compatibility functions control virus genome replication, cell-to-cell movement via plasmodesmata, and long-distance movement through the vascular system (Carrington and Whitham, 1998). Restricted infection may occur if cellular factors required by the virus are lacking or incompatible with the virus, or if the host responds to the virus by activating a defense response, such as the hypersensitive response, systemic acquired resistance, and homolog-dependent gene silencing. The genetic bases of virus-host interactions controlling susceptibility and resistance are poorly understood.

TYMV belongs to the tymovirus genus and is a plus-strand RNA virus prevalent both in cultivated and wild species of the *Brassicaceae* family of dicotyledonous plants (Morch et al., 1988, Skotnicki et al., 1992). TYMV infection in *Arabidopsis* gives rise to a symptom of yellow mosaic leaves (Bozarth et al., 1992). *Arabidopsis* is a well known model system for molecular genetic studies. To set up a TYMV-*Arabidopsis* system to study the RNA silencing antiviral pathway, TYMV whole genome under the control of an inducible promoter (**Figure 3.1**) (Aoyama and Chua, 1997) was transformed into *Arabidopsis*. Surprisingly, *Arabidopsis* plants carrying the TYMV amplicon transgene displayed the typical TYMV symptoms before transcriptional induction. The implication of this finding is two-fold. Firstly, it suggests that there is leaking transcription from this promoter as indicated previously (Zuo and Chua, 2000). Second, it suggests that viral RNA replication does not result in the activation of virus RNA silencing that is sufficient

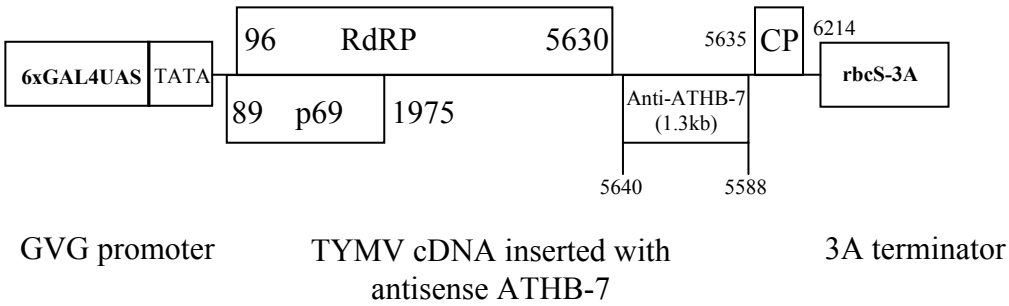


Figure 3.1 Construct of Amp-TYMV

Figure 3.1 Construct of Amp-TYMV.
Whole TYMV cDNA with a 1.3 kb insertion of antisense ATHB-7 (At2g46680) between the viral RdRP and CP genes was driven by GVG promoter (six copies of GAL4 UAS element was fused 5' to the minimal -46 to +9 region of the CaMV 35S promoter) and terminated by the pea rbcS-3A terminator. The anti-ATHB-7 was placed after the 5640 nt of TYMV cDNA. Between anti-ATHB-7 and TYMV CP, there was a duplicate sequence for nt 5588-5635 of TYMV cDNA.

to elimilate TYMV RNA in the TYMV amplicon lines. This is in contrast to the consistent virus RNA silencing associated with the PVX amplicon transgenes carried in either tobacco or *Arabidopsis* lines (Angell and Baulcombe, 1997; Dalmay et al., 2000a). It is possible that amplicon RNA silencing is inhibited by a suppressor encoded by the TYMV genome.

In this chapter, I investigated if TYMV is able to suppress either GUS transgene silencing in *Arabidopsis* L1 silencing line, or viral PVX-amplicon induced transgene silencing in Amp and GxA silencing lines.

3.2 Materials and Methods

3.2.1 Plant materials

The GFP, Amp and GxA lines (in C24 ecotype background) were obtained from David Baulcombe. They correspond to GFP142, Amp243 and GFP142 x Amp243, respectively (Dalmay et al., 2000). The GFP line contains a 35S-*GFP* transgene and Amp carries 35S-*PVX:GFP* and both lines contain a single copy of the respective transgene. The GxA line is homozygous both for 35S-*GFP* and 35S-*PVX:GFP* and is derived from a cross between GFP and Amp. PVX-*GFP* gene was silenced in Amp plants, both *PVX-GFP* and *GFP* genes were silenced in GxA plants. L1 carries a silenced *GUS* transgene (*Col-0* background) (Elmayan et al., 1998).

The TYMV amplicon lines (Amp-TYMV) were kindly provided by Nam Hai Chua and the amplicon transgene construct was subcloned from an infectious cDNA clone of TYMV (Blue Lake isolate) constructed in pCass (Ding et al., 1995). The TYMV cDNA with a 1.3 kb insertion of antisense ATHB-7 (At2g46680) between the viral RdRP and CP genes was driven by GAL4-VP16-glucocortical receptor (GVG)-inducible promoter,

which consists of six copies of GAL4 UAS element, was fused 5' to the minimal CaMV 35S promoter (-46 to +9 region) and terminated by the pea rbcS-3A terminator (**Fig. 3.1**). The construct was transformed into *Arabidopsis* Col-0. ATHB-7 is an *Arabidopsis* homeobox gene and induced by water deficit and by abscisic acid (Soderman et al., 1996).

Homozygous Amp-TYMV plants (single copy insertion line) were crossed with homozygous L1, Amp and GxA plants to create F1: L1 x Amp-TYMV, Amp x Amp-TYMV and (GxA) x Amp-TYMV.

3.2.2 RNA Analysis

Total plant RNA extraction and Northern blotting analysis were carried out as described previously (Li et al., 1999). Hybridization probes were labeled with ^{32}P using the Amersham Megaprime DNA labeling kit. GFP probe was derived from a PCR product of full length GFP. GUS probe was generated using primer pairs GUS1 (5'-ATGGTCCGTCCTGTAGAA-3') and GUS2 (5'-TGCCAGTTCAGTTCGTTGTTC-3'). siRNAs were isolated, separated by denaturing polyacrylamide gel electrophoresis (PAGE) (See section 2.18.1.1), and blotted to a nylon membrane as described (Hamilton and Baulcombe, 1999; Llave et al., 2000). The sense and antisense RNA probes for siRNAs of GFP and GUS were generated by *in vitro* transcription from the full sequences of GFP and GUS (in pBluescriptIISK$^+$ vector) in the appropriate direction with T3 and T7 promoters.

3.2.3 Plant inoculation

For plant inoculation, an infectious plasmid (Anne Mackenzie, unpublished data) containing the 35S promoter-controlled full-length TYMV-BL cDNA (Skotnicki *et al.*, 1992) in the pCass vector as described (Ding et al., 1995b) was used to inoculate Chinese

cabbage plantlets. Sap of TYMV infected Chinese cabbage leaves (in 100 x dilution) was used to inoculate *Arabidopsis* plants with 6 to 8 leaves at approximately 4 weeks. After 14 days post-inoculation, systemic leaves with disease symptoms were sampled for total RNA extraction.

3.2.4 GFP imaging

Amp and GxA *Arabidopsis* plants after 14 days post-inoculation of TYMV, Amp x Amp-TYMV and (GxA) x Amp-TYMV F1 plants at about 4 weeks were used for GFP imaging as described in Section 2.15.

3.2.5 GUS staining

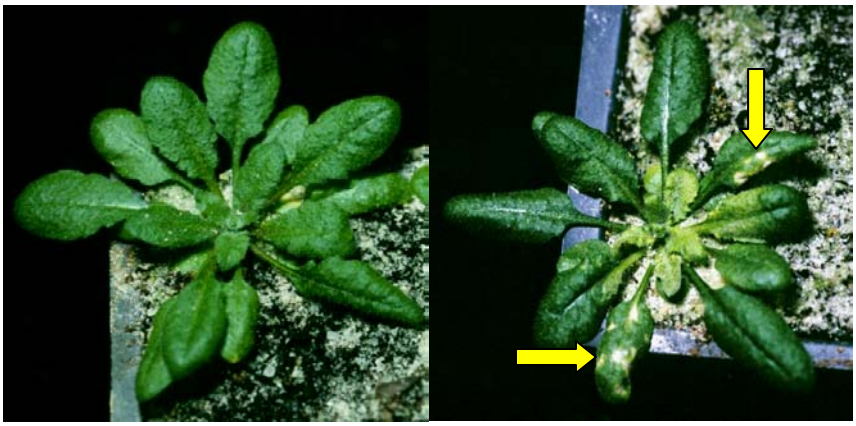
L1 plants after 14 days post-inoculation of TYMV, and L1 x Amp-TYMV F1 plants at about 4 weeks were used for histochemical localization of β-glucuronidase (GUS) activity as described by Jefferson (1987) (See Section 2.15).

3.3 Results and discussion

3.3.1 Transgenic TYMV amplicon causes disease symptoms in *Arabidopsis* plants

In *Arabidopsis* (ecotype Col-0), TYMV induces necrosis in inoculated leaves and yellow mosaic symptoms in systemically infected leaves (**Figure 3.2 A,** plant on the right). The TYMV amplicon plants also showed obvious viral symptoms of yellow mosaic leaves

A

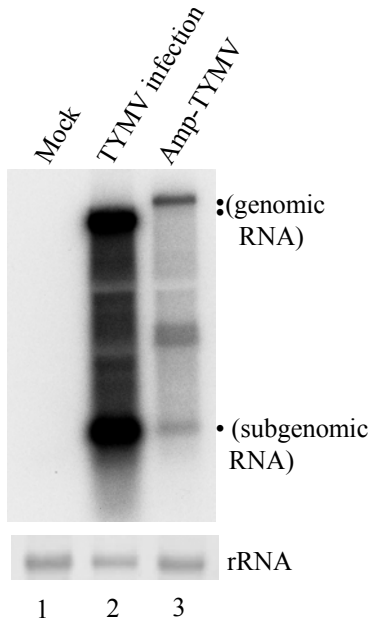


Col-0 uninfected Col-0 TYMV infected

B

1 2 3

C



D

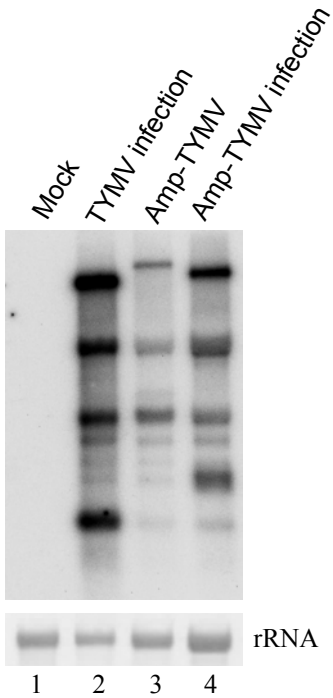


Figure 3.2

Figure 3.2 Amp-TYMV transgenic *Arabidopsis* Col-0 plant shows disease symptoms.

A. Necrosis on *Arabidopsis* leaves inoculated with TYMV: the plant on the left : wild type mock control; the plants on the right: TYMV infected plant (10 dpi). The two yellow arrows indicate the inoculated leaves.

B. Disease symptoms: plant 1: an *Arabidopsis* (Col-0) plant inoculated with TYMV (14 dpi); plant 2: wild type control; plant 3: Amp-TYMV transgenic plant.

C. The viral symptoms were associated with the expression of viral genes. Five ug total RNA was loaded in each lane. Methylene blue staining of 28S rRNA is shown as a loading control. The filters were probed with labeled DNA sequences corresponding to nucleotides 5591-6260 of TYMV genome. The amplicon transgene contained an insertion of non-viral sequence (1.3kb) upstream of the viral CP gene and thus gave rise to a recombinant viral genomic RNA longer than the wt TYMV genomic RNA although the size of the CP subgenomic RNA remained unaltered.

D. Northern blot showing that the non-viral sequence did not prevent viral replication. The sample from systemic leaves infected with Amp-TYMV leaf saps was loaded in lane 4. The coat protein gene was used as probe.

without DEX induction, although the symptoms were not as severe as those caused by TYMV inoculation (**Figure 3.2 B,** plant on the right). Attempts to transform *Arabidopsis* with the TYMV cDNA controlled under the full-length 35S promoter (35S-TYMV) were not successful, resulting in only one transformant that was very small with strong severe disease symptoms and did not produce any seeds. This suggests that the base-line, leaky transcription of the TYMV RNA from the GVG promoter is essential to ensure the viability of the amplicon plants. Northern blot hybridizations showed that the Amp-TYMV transgenic plants contained much lower levels of viral RNAs than Wt plants infected with TYMV by mechanical inoculations (**Figure 3.2 C**), indicating a correlation between virus accumulation and symptom severity.

Back inoculation to Wt plants using sap from Amp-TYMV transgenic plants produced near wild type TYMV symptoms, indicating the accumulation of infectious TYMV in the Amp-TYMV plants, hybridizations detected a genomic RNA in the infected plants (Lane 4, **Figure 3.2 D**) with a size larger than the Wt TYMV (Lane 2), but smaller than the Amp-TYMV (Lane 3). RT-PCR and sequencing analysis showed that most of the non-viral insert in the Amp-TYMV was deleted in the progeny virus.

3.3.2 Suppression PTGS by TYMV inoculation

The observation that the Amp-TYMV transgene led to virus accumulation and symptom induction suggests that TYMV may suppress PTGS. To test this hypothesis, three *Arabidopsis* silencing lines, L1, Amp and GxA, were inoculated with TYMV. L1 carries a silencing sense transgene encoding GUS in a single locus (Elmayan and Vaucheret, 1996). Four PTGS mutants, *sgs2* (RdRP), *sgs3*, *hen1* and *ago1* were derived from this line

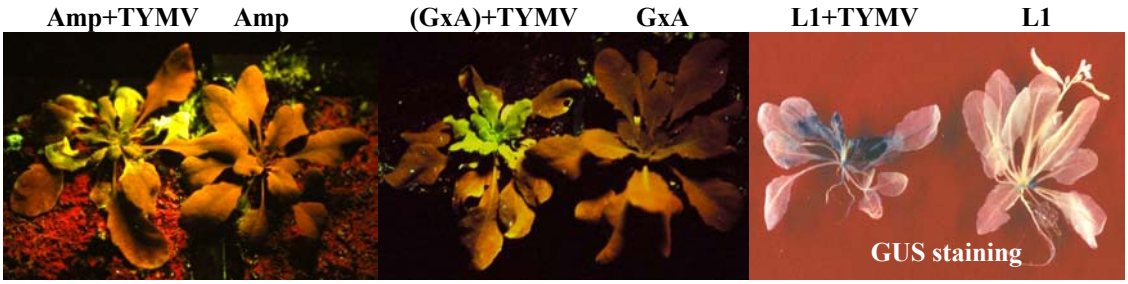
(Mourrain et al., 2000e; Fagard et al., 2000; Boutet et al., 2003). The Amp line contains the whole PVX genome (called Amplicon or Amp) in which *GFP* is inserted (Dalmay et al., 2000a). PVX-GFP is silenced in Amp line, and because of the homology between PVX:GFP and the GFP transgene, the GFP gene is also silenced in the GxA line. Two PTGS mutants *sde1* (*RdRP*/*sgs2*) and *sde3* (RNA helicase) were derived from this GxA silencing line (Dalmay et al., 2000b; Dalmay et al., 2001).

Green fluorescence and GUS staining analyses showed that the GUS activity in the L1 line and GFP activity in Amp and GxA lines were restored after infection with TYMV (**Figure 3.3 A**, plant 1, 3, 5). TYMV suppression of PTGS was further confirmed by Northern blot detection of the mRNA for GUS, GFP and of the PVX:GFP genomic and subgenomic RNAs in TYMV-infected plants (**Figure 3.3 B**, *GFP*; **Figure 3.3 C**, *GUS*). Notably, reversal of PTGS by TYMV infection occurred only in those leaves that had newly emerged, similar to PTGS suppression by CMV2b, TAV2b and TBSV-p19 (Li and Ding, 2001).

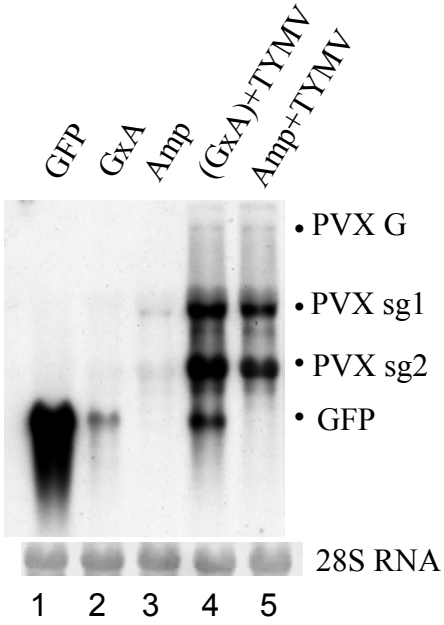
3.3.3 Suppression of PTGS by the TYMV amplicon transgene

To determine if the recombinant TYMV derived from the TYMV amplicon transgene was able to suppress PTGS, the Amp-TYMV line was crossed with each of the three silencing lines. Analyses of the F1 plants of Amp x Amp-TYMV, (GxA) x Amp-TYMV and L1 x Amp-TYMV revealed a strong suppression of PTGS as indicated by the restoration of the GUS activity and GFP activity in respective F1 plants (**Figure 3.4 A**, plant 1, 3, 5). RNA blot hybridizations showed that levels of *GUS*, *PVX-GFP* and *GFP* transcripts were significantly enhanced (**Figure 3.4 B**, GFP; **Figure 3.4 C**, GUS) in the F1

A


Amp+TYMV
Amp
(GxA)+TYMV
GxA
L1+TYMV
L1
GUS staining
1
2
3
4
5
6

B


GFP
GxA
Amp
(GxA)+TYMV
Amp+TYMV
• PVX G
• PVX sg1
• PVX sg2
• GFP
28S RNA
1 2 3 4 5

C

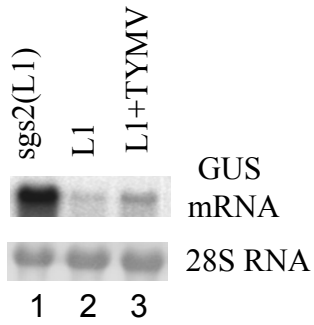

sgs2(L1)
L1
L1+TYMV
GUS mRNA
28S RNA
1 2 3

Figure 3.3 Suppression of PTGS by TYMV infection.

A. Restoration of GFP and GUS activities in silenced lines by TYMV infection (examined 14 dpi). Plant 1: Amp (GFP); plant 3: GxA (GFP); plant 5: L1 (GUS) were infected with TYMV. GFP fluorescence was detected under UV light. GUS activity was detected by GUS staining method. Plants 2, 4 and 6 are respective mock inoculated controls.

B&C. The restoration of GFP and GUS activities by TYMV infection was correlated with the restoration of the expression of the silenced PVX-GFP, *GFP* and *GUS* genes, respectively. B: full-length *GFP* probe for Amp and GxA lines; C: *GUS* probe for L1 line. GFP expression line 35S-*GFP* and GUS expression line *sgs2*/L1 were used as positive controls. 5µg total RNA was loaded in each lane. Methylene blue staining of 28S rRNA is showing as a loading control.

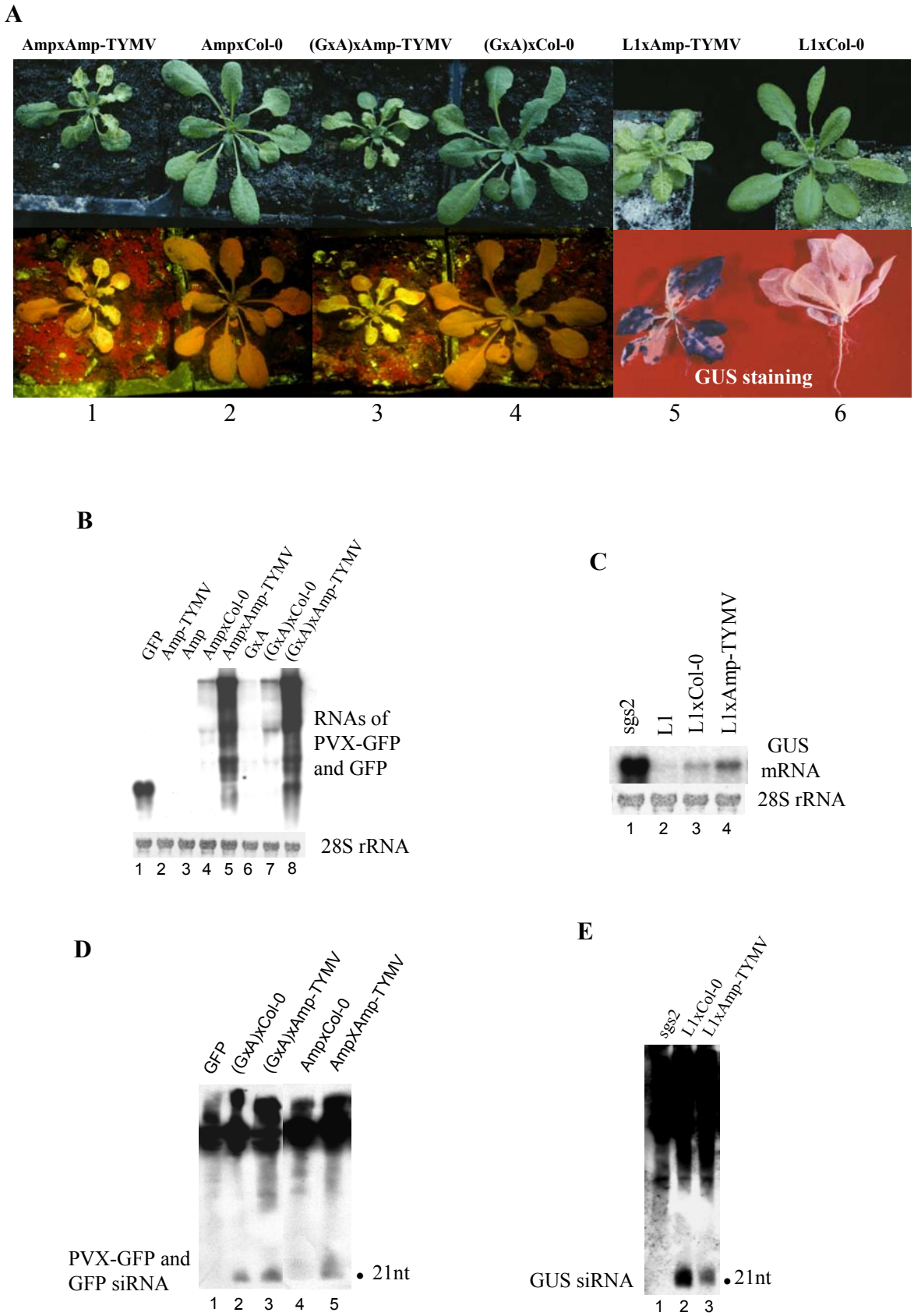

A
AmpxAmp-TYMV
AmpxCol-0
(GxA)xAmp-TYMV
(GxA)xCol-0
L1xAmp-TYMV
L1xCol-0
GUS staining
1 2 3 4 5 6
B
GFP
Amp-TYMV
Amp
AmpxCol-0
AmpxAmp-TYMV
GxA
(GxA)xCol-0
(GxA)xAmp-TYMV
RNAs of PVX-GFP and GFP
28S rRNA
1 2 3 4 5 6 7 8
C
sgs2
L1
L1xCol-0
L1xAmp-TYMV
GUS mRNA
28S rRNA
1 2 3 4
D
GFP
(GxA)xCol-0
(GxA)xAmp-TYMV
AmpxCol-0
AmpxAmp-TYMV
PVX-GFP and GFP siRNA
21nt
1 2 3 4 5
E
sgs2
L1xCol-0
L1xAmp-TYMV
GUS siRNA
21nt
1 2 3

Figure 3.4

Figure 3.4 Suppression of PTGS by Amp-TYMV

A. Restoration of GFP and GUS activities by Amp-TYMV transgene. Plant 1: Amp (GFP); plant 3: GxA (GFP); plant 5: L1 (GUS) crosses with Amp-TYMV. Plants 2, 4 and 6 are respective controls. Throughout the text, maternal parent was listed first in the names of F1 progeny such as Amp x Amp-TYMV.

B&C. The restoration of GFP and GUS activities was correlated with the restoration of expression of the silenced GFP and GUS genes, respectively. B : GFP DNA probe for Amp and GxA lines; C: *GUS* DNA probe for L1 lines.

D&E. The suppression of PTGS by TYMV altered the accumulation of siRNAs in those three silenced lines. ^{32}P-labeled RNA transcripts of GFP and GUS minus strains were used as probe to detect *PVX-GFP* and *GFP*, and *GUS* siRNAs respectively. Twenty μg of low molecular weight RNA was loaded for each lane.

plants, respectively, when compared with the RNA levels in the control F1 plants of AmpxCol-0, (GxA)xCol-0, and L1xCol-0. As expected, the accumulation of the GUS-specific siRNA, a hallmark of PTGS was greatly reduced in L1xAmp-TYMV F1 plants, as compared to that in L1xCol-0 (**Figure 3.4 E**). Interestingly, in AmpxAmp-TYMV or (GxA)xAmp-TYMVF1 plants, both the *GFP*-specific and PVX:GFP specific siRNAs accumulated to higher levels than those in the control plants despite TYMV suppression of the PVX amplicon silencing (**Figure 3.4 D**). The data presented collectively demonstrate that TYMV suppresses PTGS in *Arabidopsis* and thus is likely to encode a suppressor of PTGS.

Chapter 4

TYMV p69 suppresses PTGS at the upstream of dsRNA synthesis

4.1 Introduction

In plants, PTGS can be induced by transgenes that encode either a highly transcribed sense RNA or an inverted-repeat RNA (IR-RNA) that is folded into a long dsRNA. Identification of genetic components of PTGS showed that related genes are required for gene silencing in filamentous fungi, plants and animals (Hutvagner and Zamore, 2002). Up to date, 5 *Arabidopsis* genes have been identified to be required for sense transgene induced gene silencing. *SGS2/SDE1* is a putative RdRP (Mourrain et al., 2000; Dalmay et al., 2000b). *AGO1* belongs to the Argonaute gene family (Fagard et al., 2000). *SDE3* is homologous to RNA helicase (Dalmay et al., 2001). *SGS3* and *HEN1* both are unique to plants and have no similarity with any known protein (Mourrain et al., 2000; Boutet et al., 2003). *AGO4* was also reported to be related with the PTGS pathway, as *ago4* knockout mutation can block histone and DNA methylation and the accumulation of 25 nt siRNA corresponding to the retroelement *AtSN1* (Zilberman et al., 2003). Interestingly both *SGS2/SDE1* and *SDE3* are not required for virus induced gene silencing (VIGS) (Dalmay et al., 2000a; Dalmay et al., 2001) and recent studies showed that *SGS2/SDE1, SGS3* and *AGO1* are all dispensable for IR-RNA induced silencing (Beclin et al., 2002). Considering all these results, it is proposed that the pathway to make dsRNA in plants is branched and all branches of the pathway converge at dsRNA.

A biological function for RNA silencing, first established in plants, is as an adaptive defense against RNA and DNA viruses (Lindbo *et al*., 1993; Ratcliff *et al*., 1997; 1999; Covey *et al*., 1997; Al Kaff *et al*., 1998; Waterhouse *et al*., 2001). A recent study has demonstrated a similar antiviral role for RNA silencing in the animal kingdom using a *Drosophila* model (Li *et al*., 2002, Li *et al*., 2004). The antiviral response may be triggered by virus-specific dsRNA produced during genome replication, by transcription from converging promoters, or through recognition of viral RNAs by host RdRP, as proposed in plants (Mourrain *et al.*, 2000; Voinnet *et al.*, 2000; Dalmay *et al.*, 2000b; Szittya *et al*., 2002). As a counter-defense, viruses encode proteins such as HC-Pro and Cmv2b that suppress RNA silencing at various steps in the pathway (Brigneti *et al*., 1998; Kasschau and Carrington, 1998; Anandalakshmi *et al*., 1998; Beclin *et al*., 1998; Voinnet *et al*., 1999; Li *et al*., 1999; Mlotshwa *et al*., 2002; Silhavy *et al*., 2002). The identification of a silencing suppression function typically provides a molecular basis for previously observed defects in viral accumulation and spread of virus mutants that lack a functional suppressor (Li and Ding, 2001; Mlotshwa *et al.*, 2002).

As described in Chapter 3, TYMV was able to suppress RNA silencing induced by either a sense RNA or a viral amplicon. In this chapter, the TYMV protein involved in suppression of PTGS was firstly identified. The mechanism of PTGS suppression by the TYMV protein was analysed by both molecular and genetic approaches. The results show that TYMV encodes a unique PTGS suppressor that targets a step upstream of dsRNA synthesis mediated by the host cellular RdRP.

4.2 Materials and methods

4.2.1 Materials

Arabidopsis lines used in this study were L1 (GUS silencing line), Amp (*PVX-GFP* silencing line), GFP (*35S-GFP* expression line), GxA (silencing line), as well as wild type ecotypes C24, Col-0, Ws-0.

Line *IRRbx1* [inverted-repeat *Rbx1* (*Ring Box 1*) silencing line]: The heterozygous seeds of *IRRbx1* line (WS background) were originally obtained from Lechner and Genschick from Inst Biol Mol Plantes, France (Xu et al., 2002).

p69 (p69 overexpression line), *IRPDS* [inverted-repeat *PDS* (*phytoene desaturase*) silencing line] and *IRGFP* (inverted-repeated *GFP* silencing line) were generated as follows:

To generate p69 transgenic plants, the *p69* coding sequence of TYMV-Blue Lake (Skotnicki et al., 1992) was cloned into the binary vector pCambia1301 to replace the existing GUS coding sequence between the 35S promoter and NOS terminator, giving rise to 35S-P69 (**Figure 4.1**). The start codon (ATG) of the viral RdRP, which is four nucleotides downstream of the start codon for p69, was mutated to ACG in 35S-P69. As a result, the RdRP ORF is unlikely to be translated from 35S-P69 as the next Met codon in the RdRP ORF is 95 codons downstream. A single G to T substitution converting the 46th codon of ORF P69 into a stop codon (TAA) was introduced to create 35S-ΔP69. 35S-P69 and 35S-ΔP69 were transferred into *Arabidopsis* ecotype C24 as described (Clough & Bent, 1998).

The IR-RNA transgene targeting *PDS* (Acc. No. At4g14210) contained an inverted repeat corresponding to nucleotides 128 to 532 of the PDS mRNA. The IR-RNA targeting GFP corresponded to nucleotides 360-716 of the GFP mRNA. Inserted between the repeats of either PDS or GFP was the third intron of *Arabidopsis* actin gene 11

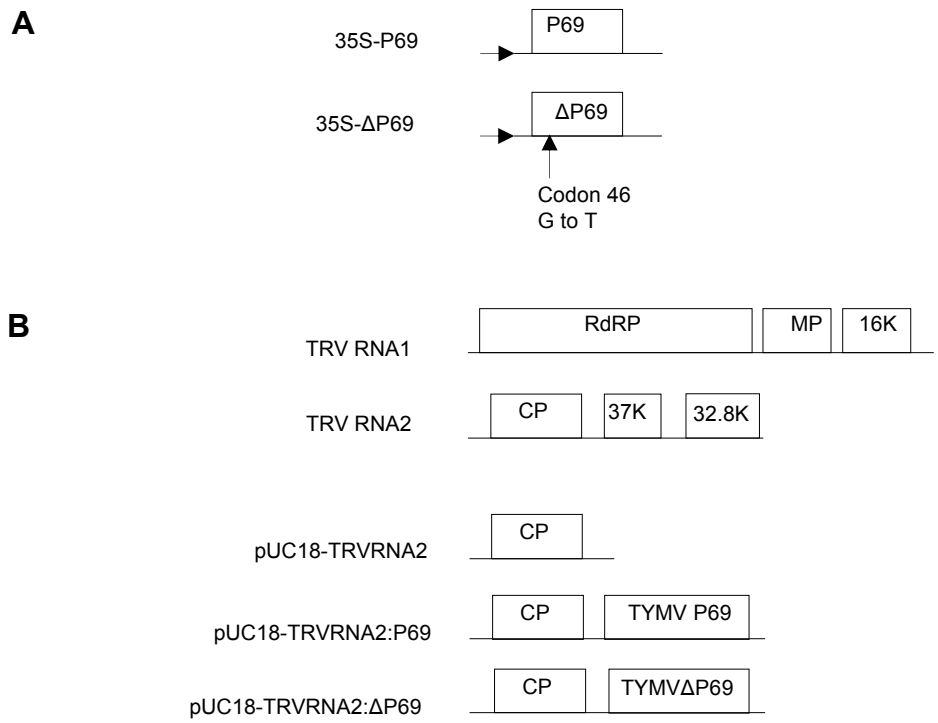

A
35S-P69
P69
35S-ΔP69
ΔP69
Codon 46
G to T
B
TRV RNA1
RdRP
MP
16K
TRV RNA2
CP
37K
32.8K
pUC18-TRVRNA2
CP
pUC18-TRVRNA2:P69
CP
TYMV P69
pUC18-TRVRNA2:ΔP69
CP
TYMVΔP69
C
GVG-IRRbx1
1xbR
GFP
Rbx1
35S-IRGFP
GFP
intron
PFG
35S-IRPDS
PDS
intron
SDP

Figure 4. 1

Figure 4.1. Diagrams showing the Constructs Used in This Work.

A. *p69* was cloned into the binary vector pCAMbia1301 by replacing the *GUS* gene. The start codon (ATG) of the viral RdRP, which is four nucleotides downstream of the start codon for p69, was mutated to ACG in 35S-P69. As a result, the RdRP ORF is unlikely to be translated from 35S-P69 as the next Met codon in the RdRP ORF is 95 codons downstream. A single G to T substitution converting the 46th codon of ORF P69 into a stop codon (TAA) was introduced to create 35S-ΔP69.

B. TRV is composed of two genomic RNAs: RNA1 and RNA2. cDNA of RNA2 was cloned in the pUC 18 vector. Both *p69* and *ΔP69* were cloned into RNA2 to replace 37k and 32.8k genes, meanwhile RNA2 containing only CP was used as a control.

C. Inverted-repeat full length Rbx1 spanned by GFP was driven by GVG promoter (Xu et al., 2002) in the pTA7002 vector (Aoyama and Chua, 1997). The IRPDS contained an inverted repeat corresponding to nucleotides 128 to 532 of the PDS mRNA. The IRGFP corresponded to nucleotides 360-716 of the GFP mRNA. Inserted between the repeats of either PDS or GFP was the third intron of *Arabidopsis* actin gene 11 (nucleotides 1957 to 2111, Acc. No. ATU27981). The PDS and GFP IR-RNA cassettes were cloned into pCambia1301 between 35S promoter and Nos terminator and transformed into Wt Col-0 and line G (Dalmay *et al.*, 2000a), respectively.

(nucleotides 1957 to 2111, Acc. No. ATU27981). The PDS and GFP IR-RNA cassettes were cloned into pCambia1301 between 35S promoter and Nos terminator (**Figure 4.1**) and transformed into Wt Col-0 and line G (Dalmay et al., 2000a), respectively. Transformants were selected on medium supplemented with hygromycin at 20 mg/L. Lines containing a single copy transgene were selected according to a hygromycin resistance segregation ratio at 3:1 of their selfed progenies. Five homozygous lines for *35S-P69* (P69b, P69c, P69d, P69h, P69i) and two for *35S-ΔP69* (ΔP69a and ΔP69b) were crossed with line GxA (as the maternal parent), while P69c was crossed with line A. In addition, P69c and P69d were crossed with L1, one heterozygous line for *IRRBX1* and one line heterozygous for IRPDS-2 with photo-bleaching phenotype was also crossed with P69c. 22 G x *IRGFP* lines that were red fluorescent under UV were obtained, 7 of which contained a single copy of the *35S-IRGFP* transgene. All hygromycin resistant progenies were red fluorescent, indicating that the silencing *IRGFP* transgene was dominant.

Aradidopsis mutants used in the study were *sde1* (an RdRP loss of function mutant in GxA genetic back ground), *sgs2* [the RdRP (same as sde1) loss of function mutant in L1 genetic background].

4.2.2 RNA Analysis

Alalysis high and low molecular weight RNAs was performed as described in Section 3.2.2.

4.2.3 Inoculation of TRV and its Derivatives

TRV2 cDNA clone TRV2-pK20GFPc (TRV coat protein + *GFP* genes were cloned into pUC18 vector) was kindly provided by Dr Stuart Macfarlane from the Scottish Crop Research Institute, U.K.. *GFP* in TRV2-pK20GFPc was removed to generate TRV

RNA2 construct or replaced by TYMV P69 or ΔP69 gene to generate TRV RNA2:*P69*/ *ΔP69* constructs, respectively (**Figure 4.1**). The *ΔP69* was the same as *ΔP69* in 35S- *ΔP69*. These constructs were used to generate TRV RNA2 strand using T7 RNA polymerase. Since TRV RNA1 can infect *Nicotiana clevelandii* alone and cause mottling symptoms on the upper leaves, the TRV RNA1 was harvested from the upper leaves. TRV RNA1 was mixed with *in vitro* transcribed TRV RNA2 in a ratio of 4 to 1 and 5 ug of the RNA mixtures were used to inoculate each *Arabidopsis* plant.

4.2.4 *Agrobacterium* infiltration in N. benthamiana

Transgenic *N. benthamiana* 16C carrying 35S-GFP was obtained from David Baulcomb at John Innes Center U.K. (Brigneti et al., 1998). The 35S-GFP and 35S-Cmv2b were constructed in pCambia1300 binary vector as described previously (Guo et al., 2002). The construction of pCAMbial:35S-*P69/ΔP69* (**Figure 4.1**) was described as above. The leaf infiltration of *A. tumefaciens* strains was performed as described previously in Section 2.14 (Brigneti et al., 1998). For the co-infiltration experiments, *Agrobacterium* culture containing 35S-*GFP* (OD_{600} =1.0) was mixed with equal volumes of 35S-*P69*, 35S- *ΔP69* and 35S-*2b* (all OD_{600} =2.0), respectively.

4.2.5 DNA Analysis

To confirm the presence of the *IR-RBX1* transgene, PCR primer pairs RB1 (in the TATA box region of GVG promoter: 5'-CATTTGGAGAGGACACGCT-3') and RB2 (at the 3' end of 1xbR – the first part of IRRbx1: 5'-AAGCCTCCAGCAGCGTAGC-3') were used to amplify the corresponding DNA fragment (~450 bp).

The analysis of DNA methylation of the *GFP* coding sequence was carried out as described (Jones et al., 1999). Genomic DNA (30 ug of each sample) was digested with

HaeIII and hybridized with a ^{32}P-labeled DNA probe corresponding to the entire GFP coding sequence. Methylation of the *GUS* coding sequence was analyzed as described (Mourrain et al., 2000b). Genomic DNA 20 ug of each sample was digested with *HpaII* or *MspI* and detected by a probe derived from a 1.7 kb fragment of *GUS* coding sequence. The same membrane was stripped and hybridized with a 3'-portion of *SPL3*-specific probe to confirm that the digestions were complete as there is only one *HpaII*/*MspI* recognition sequence (CCGG) within the *SPL3* genomic DNA.

4.3 Results

4.3.1 p69 Suppresses PTGS in Tobacco

TYMV contains three genes, two of which are initiated from out-of-frame overlapping reading frames encoding the viral *RdRP* and *p69*, and are translated from the genomic RNA. In contrast, the coat protein is produced from a subgenomic RNA that is 3' co-terminal with the genomic RNA. It is likely that the observed PTGS suppression activity of TYMV is encoded by *p69*, based on its role in viral spread and virulence (Bozarth et al., 1992; Tsai et al., 1993), which is similar to the known viral suppressors that are disease determinants but not required for virus replication (Li and Ding, 2001). p69 protein appears unique to tymoviruses and does not share significant homology to any known proteins in the database.

The co-infiltration assay established in the 16C *N. benthamiana* GFP plants was firstly used to test whether *p69* could suppress PTGS. *p69* gene was cloned into the binary vector pCambia1301 so that the expression of *p69* is under the control of CaMV 35S promoter (**Figure 4.1 A**). An early translational truncation mutant Δ*P69* was generated as a negative control (**Figure 4.1A**). An *A. tumefaciens* strain carrying a binary plasmid that

encodes 2b protein of CMV was used as a positive control (Guo et al., 2002). *A. tumefaciens* strains containing 35S-*P69*, 35S-Δ*P69* and 35S-*2b* plasmids were respectively mixed with another strain containing 35S-*GFP* plasmid, which is used to initiate the silencing of the *GFP* transgene, and co-infiltrated into two fully-expanded leaves of each *N. benthamiana* 16C plant. Meanwhile, *Agrobacteria* containing pCambia1301 plasmid alone was infiltrated as a control.

As expected, PTGS was induced when 35S-*GFP* alone was infiltrated onto 16C leaves and GFP expression was systemically silenced in all 30 plants tested at 9 days post infiltration (dpi) (**Table 4.1**). However, the systemic silencing of *GFP* transgene was totally blocked in 29 out of 30 plants when 35S-*2b* was co-infiltrated with 35S-*GFP* (**Table 4.1**). On the other hand, when observed under UV-light, systemic silencing was only delayed by 1 to 2 days when 35S-*P69* was co-infiltrated with 35S-*GFP*, with 23 of 30 plants infiltrated showing systemic GFP silencing at 9 dpi. However, all plants showed systemic silencing at 15 dpi (**Figure 4.2**, **A**, middle plant). All 30 plants co-infiltrated with 35S-Δ*P69* displayed no visible suppression of PTGS (**Figure 4.2**, **A**, right plant; **Table 4.1**) and did not show much difference from those infiltrated with 35S-GFP alone.

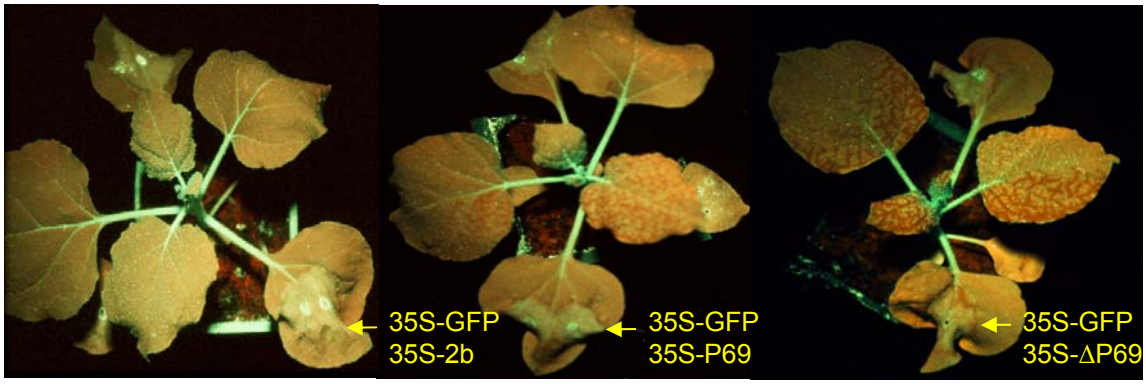
Local GFP silencing in the infiltrated leaves was further analyzed. There were not much difference in local silencing between 35S-*GFP* infiltration alone and co-infiltration with 35S-*GFP* and 35S-Δ*P69*. In the first two days after infiltration, the green fluorescence at the infiltration sites was increased significantly under UV light, because of the transient GFP expression from the infiltrated 35S-GFP. From 3 dpi, green fluorescence within infiltrated patches started to decrease, and a red fluorescent ring around the infiltrated patches could be seen from 4 dpi (**Figure 4.2 A**, right yellow arrow). Molecular analysis confirmed the fluorescent observation. The GFP-specific siRNAs accumulated to

Table 4.1

	Total plants	Systemic GFP silencing
Vector	30	0
35S-GFP	30	30
35S-GFP+ 35S-P69	30	23
35S-GFP+ 35S-ΔP69	30	30
35S-GFP+ 35S-2b	30	1

Table 4.1 Comparison of systemic GFP silencing in *N. benthamiana* coinfiltration with different constructs at 9 dpi.

A


35S-GFP
35S-2b
35S-GFP
35S-P69
35S-GFP
35S-ΔP69

B

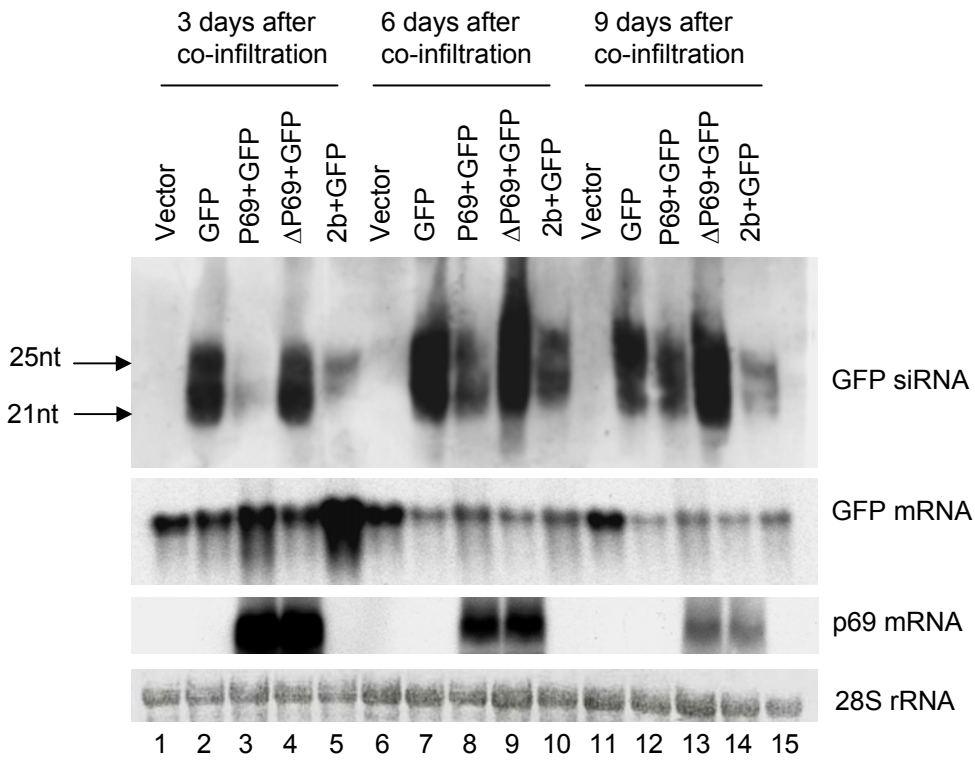

3 days after co-infiltration
6 days after co-infiltration
9 days after co-infiltration
Vector
GFP
P69+GFP
ΔP69+GFP
2b+GFP
25nt
21nt
GFP siRNA
GFP mRNA
p69 mRNA
28S rRNA
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Figure 4.2

Figure 4.2 TYMV p69 Suppresses PTGS in Tobacco.

A. p69 causes subtle but significant suppression of PTGS in *N. benthamiana*. 35S-GFP was infiltrated into the leaf of GFP expressing line 16C alone (data not shown; Guo et al., 2002), or co-infiltrated with 35S-P69 (middle plant) or with 35S-CMV2b (as a positive control) (left plant) or with 35S-ΔP69(as a negative control) (right plant). GFP systemic silencing in the treatment of 35S-GFP+35S-P69 was delayed (less red) compared to the control of 35S-GFP+35S-ΔP69. Photo was taken 9 dpi under UV light to visualize the GFP green fluorescence. Summary of this experiment was recorded in Table 1.

B. Levels of *GFP* specific siRNA in GFP coinfiltrated with both CMV2b (lanes 5, 10, 15) and TYMV *p69* (lane 3, 6, 9) plants were significantly reduced compared to those in the negative control (35S-GFP+35S-ΔP69) (lanes 4, 8, 12). Top panel: detection of the *GFP* specific siRNA (20 µg of low molecular weight RNA loaded); 2nd panel: confirmation of the expression of the *GFP* gene (5 µg total RNA loaded); 3rd panel: confirmation of the expression of *p69* gene using *p69* specific probe; lower panel: methylene blue staining of 28S rRNA as loading control of high molecular weight RNA blot. RNA samples were obtained at 3, 6 and 9 dpi, respectively.

very high levels in leaves infiltrated either alone or together with 35S-ΔP69 (**Figure 4.2**, **B**, top panel, lane 2, 4, 7, 9, 12, 14). Consistently, GFP mRNA levels gradually decreased from 3 to 9 dpi (**Figure 4.2**, **B**, the second panel, lane 2, 4, 7, 9, 12, 14). In the leaves infiltrated with the empty vector, green fluorescence remained unchanged, GFP siRNAs were undetectable (**Figure 4.2 B**, top panel, lane 1, 6, 11), and GFP mRNA remained at the same level from 3 dpi to 9 dpi (**Figure 4.2 B**, second panel lanes 1, 6, 11). In contrast, expression of p69 in the infiltrated leaves led to elevated levels of GFP mRNA and reduced accumulation of GFP siRNAs as compared to expression of ΔP69. This difference was most significant at 3 dpi and diminished at 9 dpi, unlike suppression by CMV2b. The results indicated that TYMV p69 acted as a suppressor of PTGS although its activity is weak and transient as compared to CMV 2b.

4.3.2 Suppression of PTGS in *Arabidopsis* by p69 expressed from a recombinant TRV

It is possible that unlike CMV 2b, p69 may not be adapted to function fully in *N. benthamiana* which is a non-host for TYMV. To test this hypothesis, a recombinant Tobacco rattle virus expressing p69 (TRV-P69) was used to infect the *Arabidopsis* silencing line homozygous for both 35S-GFP and 35S-PVX:GFP (GxA). TRV can infect *Arabidopsis* plants systemically. However, TRV-infected plants show very mild symptoms as they usually recover approximately 7 days after infection (Ratcliff et al., 2001).

TRV has two genomic RNAs and the genome organization is as shown in **Figure 4.1 B**. The coding sequence for p69 or ΔP69 was cloned into RNA2 to replace the 37K and 32.5K genes (**Figure 4.1 B**), neither of these two genes is required for TRV infection

(Ratcliff et al., 1999). GxA plants were inoculated with TRV-*P69*, TRV-Δ*P69* and TRV alone (without 37K and 32.5K genes). Seven days after GxA plants were inoculated with TRV-P69, strong green fluorescence was observed under UV light from the newly emerged leaves (**Figure 4.3 A**, plant 1). In contrast, GxA plants infected with either TRV-ΔP69 or TRV alone did not show any obvious green fluorescence (**Figure 4.3 A, plant 3, 5**). Furthermore, Northern blot hybridization detected an increase in the accumulation of both GFP mRNA and PVX:GFP genomic and subgenomic RNAs in plants infected with TRV-P69 as compared to those infected by either TRV or TRV-ΔP69 (**Figure 4.3 B, top panel**). Interestingly, p69 expression did not affect the accumulation levels of TRV RNAs (**Figure 4.3 B, middle panel**). These results collectively demonstrate that p69 expressed from TRV was an active suppressor of RNA silencing in *Arabidopsis* plants. This work also establishes the application of TRV as an expression vector to assay for reversal of silencing in *Arabidopsis* as has been demonstrated for PVX in *N. benthamiana*.

4.3.3 p69 inhibits PTGS induced by sense-RNA transgenes

To facilitate genetic studies, 35S-*P69* and 35S-*ΔP69,* which were the same constructs used in co-infiltration assays, were introduced into *Arabidopsis* (ecotype C24) plants (**Figure 4.1 A**). Transgenic plants with *35S-P69* showed TYMV disease symptom-like developmental abnormalities, in contrast 35S-*ΔP69* transformants did not (see Section 5.3.1). Homozygous lines for *35S-P69* (P69c and P69d) were crossed with L1, while C24 Wt plants were crossed with L1 plants as controls. Suppression activity in L1 silencing line showed that p69 is a potent suppressor of sense RNA-induced RNA silencing. High levels of GUS activity (**Figure 4.4 A**, plant on the left) and *GUS* mRNA (**Figure 4.4 B**) were detected in the F1 progeny plants of the crosses between line P69c and line L1. The

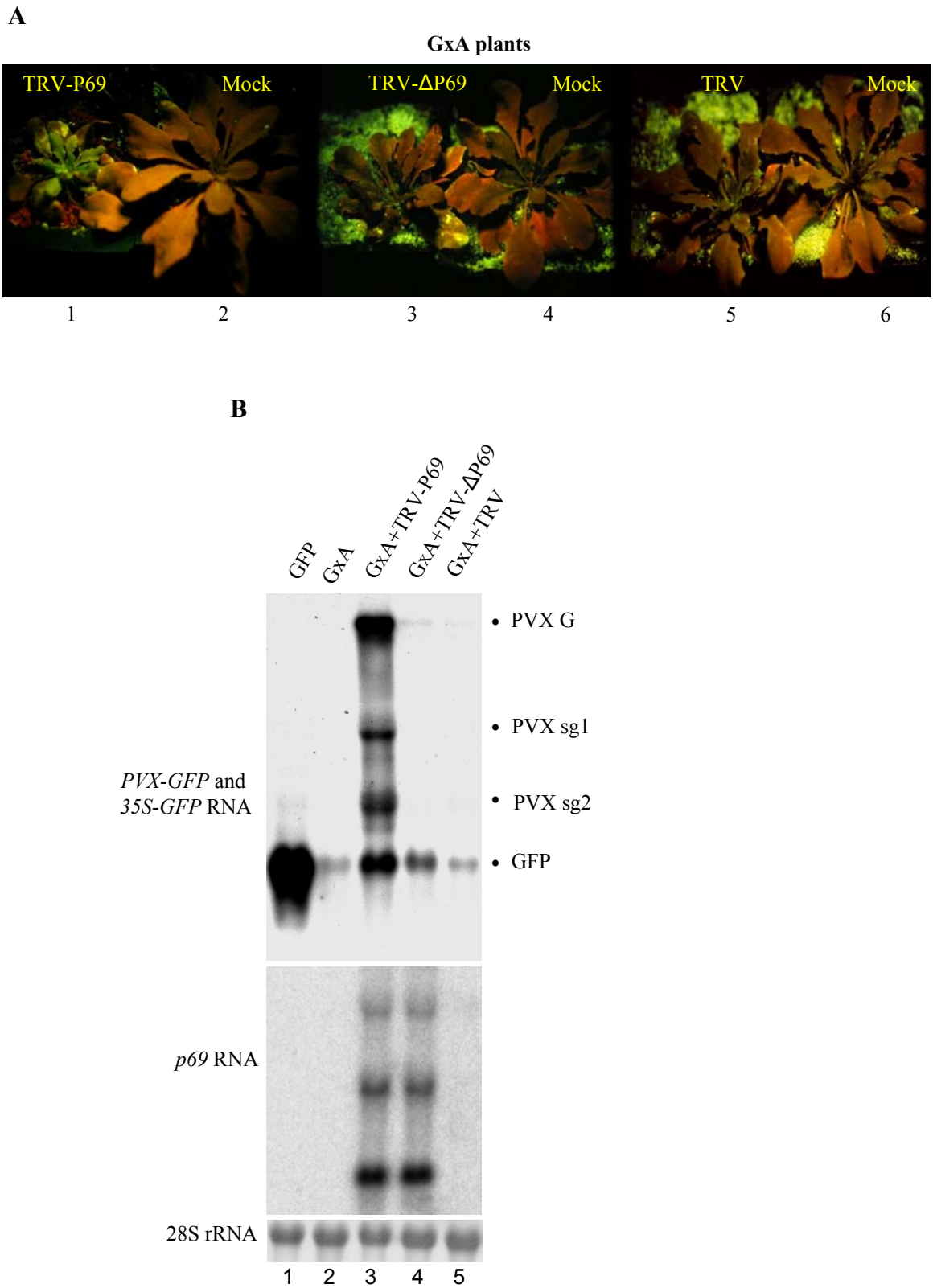

A
GxA plants
TRV-P69
Mock
TRV-ΔP69
Mock
TRV
Mock
1
2
3
4
5
6
B
GFP
GxA
GxA+TRV-P69
GxA+TRV-ΔP69
GxA+TRV
PVX G
PVX sg1
PVX sg2
GFP
PVX-GFP and
35S-GFP RNA
p69 RNA
28S rRNA
1
2
3
4
5

Figure 4.3

Figure 4.3 Suppression of PTGS in *Arabidopsis* by p69 expressed from a recombinant TRV.

A. GxA silencing plants were inoculated with TRV-P69(plant 1) ; or TRV-ΔP69 (plant 3); or TRV alone (without 37K and 32.5K genes) (plant 5). Plants: 2, 4, and 6 were mock controls. Photos were taken at 7 dpi under UV light to visualize the GFP fluorescence.

B. The restoration of the expression of GFP gene in plants inoculated with TRV-P69 was associated with the expression of PVX viral transgene. ^{32}P labeled GFP probe was used to detect PVX-GFP and 35S-GFP (top panel). The same membrane was stripped and hybridized with the p69 probe (full length gene used). Five µg total RNA was loaded at each lane. Methylene blue staining of 28S rRNA is showing as a loading control.

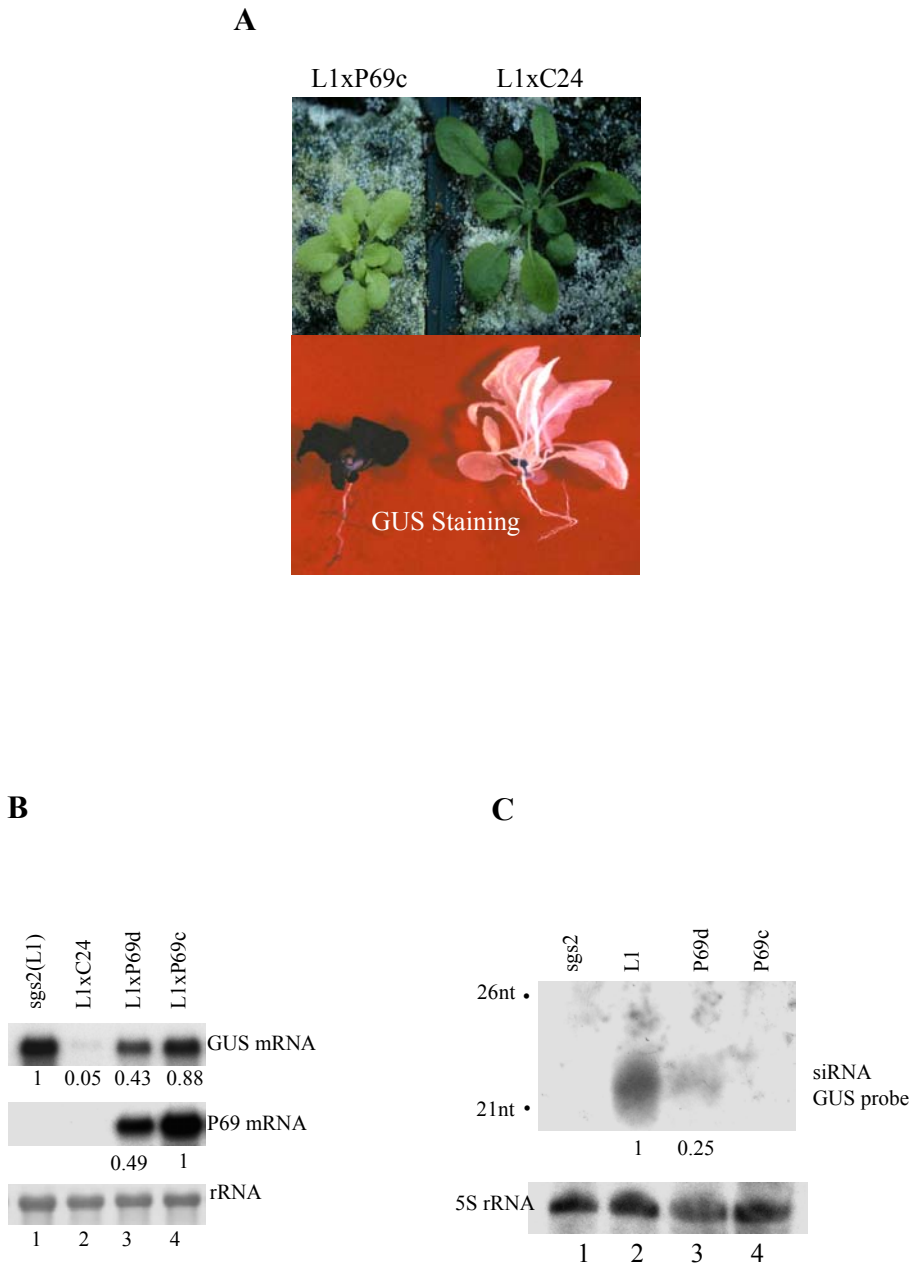

A
L1xP69c
L1xC24
GUS Staining
B
sgs2(L1)
L1xC24
L1xP69d
L1xP69c
GUS mRNA
1 0.05 0.43 0.88
P69 mRNA
0.49 1
rRNA
1 2 3 4
C
sgs2
L1
P69d
P69c
26nt
21nt
siRNA
GUS probe
1 0.25
5S rRNA
1 2 3 4

Figure 4.4

Figure 4.4 p69 inhibits PTGS induced by sense-RNA transgenes.

A. TYMV *p69* transgene strongly suppresses PTGS induced by sense-RNA transgene in *Arabidopsis*. The transgenic line of homozygous P69c was crossed with L1. F1 of the crosses of Wt (C24) x L1 were used as controls. GUS activity was restored by the P69 transgene. Plant on the left : P69c x L1 showed strong GUS staining. Note that the F1 plants were also pale and small compared with a wild type control.

B. Detection of the *GUS* mRNA in *sgs2*(L1), L1xC24, L1xP69c and L1xP69d seedlings. Probes were ^{32}P-labeled DNA corresponding to the 1.7 kb *GUS* coding sequence (Upper panel). A *p69*-specific probe was used to detect the mRNA from the *35S-P69* transgene (Middle panel). Five μg total RNA was loaded in each lane.

C. Levels of GUS specific siRNA were reduced by *p69* expression. ^{32}P-labeled RNA transcripts of *GUS* minus strand was used to detect *GUS* siRNAs. The same membrane was stripped and hybridized with the 5S rRNA probe (lower panel), which was used as a loading control. Twenty μg of low molecular weight RNA was loaded in each lane.

The mean relative accumulation (RA) level for each samples is indicated, which was determined by a computer software-Molecular Analyst®/Macintosh (Bio-RAD).

distinct GUS staining pattern, as compared to those of L1 plants after TYMV infection refer to **Figure 3.4** and F1 plants of L1xAmp-TYMV, may be contributed by the constitutive expression of *p69* from an integrated nuclear gene. In contrast, *35S-GUS* remained silenced in the F1 progeny of a similar cross between Wt and L1 (**Figure 4.4 A**, plant on the right). Furthermore, whereas the *GUS*-specific siRNAs accumulated to high levels in L1xC24 plants, they were undetectable in L1xP69c plants (**Figure 4.4 C**, upper panel, lane 4). Notably, these RNA analyses showed that suppression of *GUS* RNA silencing in P69c plants was as effective as in the *sgs2* mutant that contains a defective cellular *RdRP* (**Figure 4.4 B** and **C**, lane 1) (Mourrain *et al.*, 2000). Interestingly, silencing suppression as measured by the accumulation of both *GUS* mRNA and siRNAs, was less efficient in L1xP69d plants that expressed p69 at a lower level (compare lanes 3 and 4 of **Figure 4.4 B** and **C**). These results are consistent with those obtained from the analysis of Amp-TYMV x L1 (**Figure 3.4**), although the suppression activity was weaker in Amp-TYMV plants than in P69c and P69d, the reason for weak suppression in Amp-TYMV plants will be discussed later.

4.3.4 p69 inhibits PTGS induced by a virus-derived amplicon transgene

Viral RNAs are silenced in plants via both the cellular and viral RdRP pathways (Dalmay *et al.*, 2000b; Voinnet *et al.*, 2000). It was analyzed whether p69 suppresses PTGS targeting an amplicon transgene that encodes a replication-competent recombinant PVX:GFP in *Arabidopsis* lines Amp and GxA (Dalmay *et al*., 2000a). The autonomous silencing of the amplicon requires active replication of PVX:GFP RNA, but is *SDE1*-independent as shown in line GxA. Line G contains a highly expressed *35S-GFP*

transgene that is silenced in line GxA by the amplicon. In contrast to amplicon silencing, silencing of the *35S-GFP* transgene in line GxA requires *SDE1* and is associated with methylation of the *GFP* and *PVX:GFP* transgenes (Dalmay *et al*., 2000a).

Homozygous lines for *35S-P69* (P69b, P69c, P69d, P69h, P69i) and *35S-ΔP69* (ΔP69a and ΔP69b) were crossed with line GxA, and P69c was crossed with line Amp, while C24 Wt plants were crossed with Amp and GxA silencing plants as controls. Suppression activity in Amp and GxA silencing lines showed that p69 also suppresses PTGS mediated by a replicating viral RNA either alone or in combination with a homologous transgene. Firstly, GFP activity was highly restored in the F1 progeny of the two crosses between line P69c and both Amp and GxA lines (**Figure 4.5 A**, plant 1, 3 respectively). Northern blot hybridizations detected significant accumulation of *PVX:GFP* genomic (g) and subgenomic (sg) RNAs in both Amp and GxA plants after the *35S-P69* transgene was introduced from line P69c through genetic crosses (**Figure 4.5 B**, lanes 3 and 7, top panel). Accumulation of *GFP* mRNA was also detected in (GxA) x P69C plants (**Figure 4.5 B**, lane 7). As expected from previous work (Dalmay *et al.*, 2000b), the *GFP* mRNA and the genomic and subgenomic RNAs from PVX:GFP were detected in the *sde1*(GxA) mutant (**Figure 4.5 B**, lane 13). Interestingly, the accumulation level was lower for the *GFP* mRNA and higher for the viral RNAs in (GxA) x P69c plants than in the *sde1*(GxA) mutant. Suppression of the viral and *GFP* RNA silencing was weaker in lines (GxA) x P69b and (GxA) x P69d than in (GxA) x P69c and undetectable in (GxA) x P69h and (GxA) x P69i as in lines (GxA) x ΔP69a and (GxA) x ΔP69b. Comparison p69 mRNA levels (**Figure 4.5B**) indicates that silencing suppression activity was determined by the expression levels of the *P69* transgene and by whether or not the *p69* transgene was

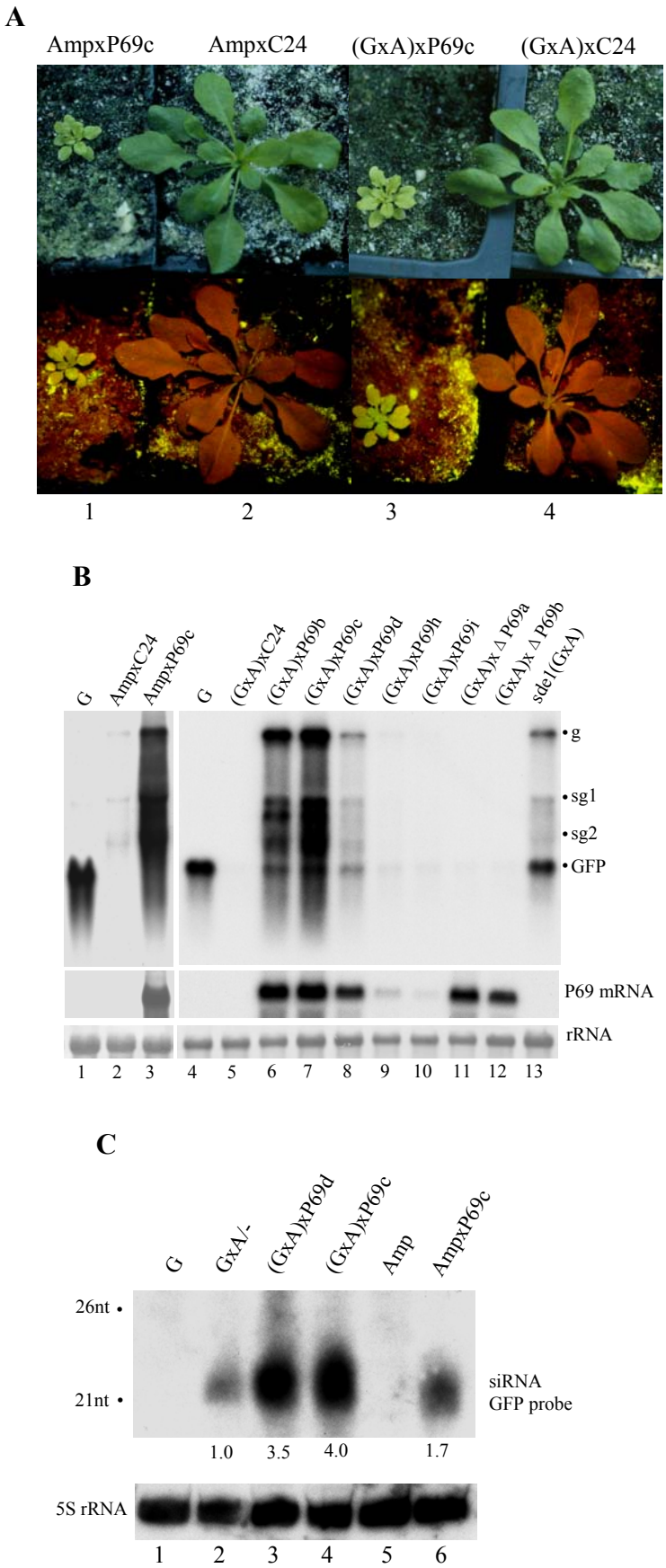

A
AmpxP69c
AmpxC24
(GxA)xP69c
(GxA)xC24
1
2
3
4
B
G
AmpxC24
AmpxP69c
G
(GxA)xC24
(GxA)xP69b
(GxA)xP69c
(GxA)xP69d
(GxA)xP69h
(GxA)xP69i
(GxA)x Δ P69a
(GxA)x Δ P69b
sde1(GxA)
• g
• sg1
• sg2
• GFP
P69 mRNA
rRNA
1 2 3 4 5 6 7 8 9 10 11 12 13
C
G
GxA/-
(GxA)xP69d
(GxA)xP69c
Amp
AmpxP69c
26nt •
21nt •
siRNA
GFP probe
1.0
3.5
4.0
1.7
5S rRNA
1 2 3 4 5 6

Figure 4.5

Figure 4.5 TYMV p69 inhibits PTGS induced by a virus-derived amplicon transgene.

A. TYMV *p69* transgene strongly suppresses PTGS induced by PVX-GFP amplicon transgene in *Arabidopsis*. The transgenic line of homozygous P69c was crossed with Amp and GxA, respectively. F1 of the crosses of C24 x Amp and x (GxA) were used as controls. GFP activity was restored by *P69* transgene. Plant 1: P69c x Amp (GFP); plant 3: P69c x (GxA) (GFP). Note that the F1 plants were also pale and small compared with a wild type control.

B. Silencing suppression by the *p69* transgene introduced into lines A and GxA by genetic crosses. Total RNA was extracted from the hemizygous F1 plants and from homozygous G and *sde1*(GxA) plants as indicated on top of each lane and analyzed for the accumulation of *GFP* mRNA and PVX:GFP RNAs as in (Upper panel). Five μg total RNA was loaded in each lane except for lanes 4-13 where 3 μg was loaded and the amount loaded was visualized by methylene blue staining of rRNA (bottom panel). ^{32}P-labelld DNA probe corresponding to the full-length GFP coding sequence was used to detect PVX-GFP and GFP RNA. The PVX-GFP subgenomic RNAs and GFP mRNA were marked with dots. The same membrane was stripped and hybridized with P69 probe to detect P69 RNA (Middle panel). An *p69*-specific probe was used to detect the mRNA from the *35S-P69* transgene.

C. Levels of GFP specific siRNA were enhanced by p69 expression. ^{32}P-labeled RNA transcripts of *GFP* minus strain was used to detect PVX-*GFP* and *GFP* siRNAs. The same membrane was stripped and hybridized with the 5S rRNA probe (lower panel), which was used as a loading control. Twenty μg of low molecular weight RNA was loaded in each lane.

translatable in these plants. Hybridizations using a probe complementary to the GFP sequence detected the presence of siRNAs in Amp x P69c and (GxA) x P69c, P69d plants (**Figure 4.5 C**). In fact, the siRNAs became more abundant in these amplicon lines after p69 was introduced (**Figure 4.5 C**, lanes 3, 4, 6). These results are also consistent with the results obtained from the analysis of Amp-TYMV x Amp and x (GxA) F1 plants (**Figure 3.4**). Since Amp x P69c plants did not carry the *35S-GFP* transgene, all of the GFP-specific siRNAs detected in these plants have likely come from the degradation of replicating PVX:GFP, indicating that viral RNA was targeted for silencing in the presence of p69. Thus, p69 suppression of virus RNA silencing led to enhanced accumulation of viral siRNA, as found in the *sde1* (GxA) mutant (Dalmay *et al.*, 2000b), rather than siRNA elimination observed in L1 x P69c plants.

p69 as an active suppressor of RNA silencing induced by and targeted against a replicating viral RNA is also consistent with the observation in Chapter 3 that a TYMV-based amplicon transgene was not silenced in transgenic plants. *Arabidopsis* plants carrying this TYMV amplicon displayed the characteristic TYMV symptoms and suppressed the GUS and Amp-PVX transgene silencing, which was unlike the PVX-based amplicon that induces consistent silencing in transgenic tobacco and *Arabidopsis* plants (Angell & Baulcombe, 1997, Dalmay et al., 2000a). Compared to the *35S-P69* transgene (**Figure 4.5**), however, suppression of *GUS* silencing by the TYMV amplicon was less effective (**Figure 3.4**); this was probably caused by silencing of the amplicon transgene as indicated by detection of TYMV-specific siRNAs in the amplicon plants (**Figure 4.6**, lower panel, lane 3) and much lower levels of the genomic and subgenomic viral RNAs in amplicon plants than in TYMV-infected plants (**Figure 4.6**, upper panel, compare lanes 2 and 3). Consistent with the findings from the PVX amplicon plants, p69 expression from

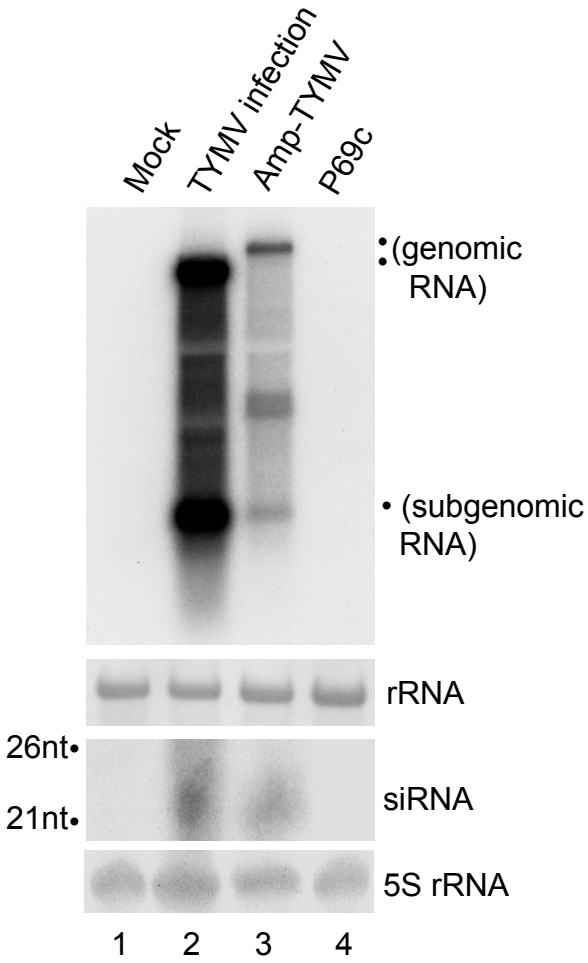

Mock
TYMV infection
Amp-TYMV
P69c
(genomic RNA)
• (subgenomic RNA)
rRNA
26nt•
21nt•
siRNA
5S rRNA
1
2
3
4

Figure 4.6

Figure 4.6 Detection of TYMV-specific high and low molecular weight RNAs in either the TYMV-based amplicon plants or wt plants infected TYMV. The filters were probed respectively with labeled DNA and RNA sequences corresponding to nucleotides 5591-6260 of TYMV genome.

TYMV also did not prevent accumulation of viral siRNAs in TYMV-infected plants (**Figure 4.6**, lower panel, lane 2).

One of the interpretations for these data is that p69 suppresses RNA silencing at a step upstream of dsRNA production so that it is unable to prevent Dicer cleavage of virus-derived dsRNAs into siRNAs and also unable to prevent *PVX:GFP* siRNAs from targeting homologous cellular mRNA for degradation.

4.3.5 p69 suppresses DNA methylation of sense-RNA silencing transgene

The coding sequences of PTGS trangenes are also frequently found to be methylated. Thus far, all PTGS mutants resulted in demethylation of PTGS transgenes (Dalmay et al., 2000b; Dalmay et al., 2001; Mourrain et al., 2000; Fagard et al., 2000; Boutet et al., 2003). On the other hand, demethylation was also found to be associated with the restoration of expression of silenced transgenes by some PTGS suppressors (Llave et al., 2000; Guo and Ding, 2002). To find out if p69 has effects on transgene methylation, the status of methylation of *GFP* and *GUS* genes in GxA and L1 lines and in F1 plants of P69c x (GxA) and x L1 was examined via genomic DNA gel blot hybridization, respectively.

As expected, the *GUS* DNA in the L1 plants was partially resistant to digestion by the methylation-sensitive restriction enzymes, *HpaII* and *MspI* (**Figure 4.7 A**, top left panel). *MspI* is an isoschizomer of *HpaII*. When the external C in the sequence CCGG is methylated, *MspI* and *HpaII* cannot cleave. However, unlike *HpaII, MspI* can cleave the sequence when the internal C residue is methylated. In contrast to the DNA in the L1 plants, the *GUS* DNA from the L1 x P69c plants, in which the *GUS* siRNAs were undetectable, was digested completely by *MspI* although the digestion by *HpaII* was less

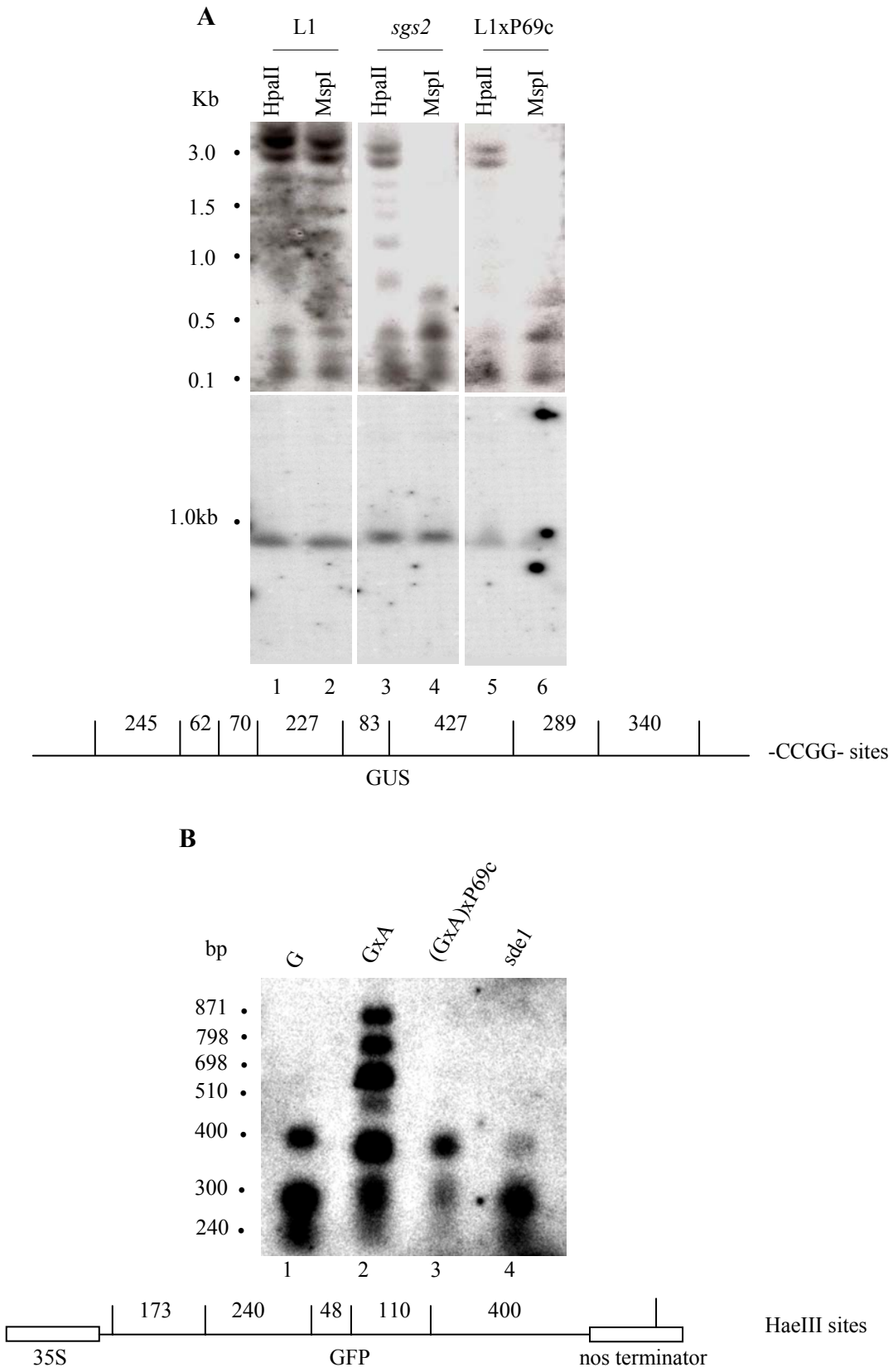

A
L1
sgs2
L1xP69c
Kb
HpaII
MspI
HpaII
MspI
HpaII
MspI
3.0
1.5
1.0
0.5
0.1
1.0kb
1 2 3 4 5 6
245 62 70 227 83 427 289 340
-CCGG- sites
GUS
B
bp
G
GxA
(GxA)xP69c
sde1
871
798
698
510
400
300
240
1 2 3 4
173 240 48 110 400
HaeIII sites
35S
GFP
nos terminator

Figure 4.7

Figure 4.7 TYMV p69 causes the demethylation of silenced GFP and GUS transgenes

A. Twenty ug genomic DNAs extracted from L1, *sgs2* and L1 x P69c were digested either with *HpaII* or *MspI*. A 1.7kb DNA fragment of *GUS* gene was used as the hybridization probe. The same filter in the top panel of (**A**) was rehybridized with a ^{32}P-labeled probe specific for 3' portion of the *SPL3* gene (middle panel of **A**) to show that the amount of DNA loaded and the relative level of restriction enzyme digestion in each lane. Both HpaII and MspI recognize DNA site (-CCGG-). But HpaII activity can be blocked by methylation at either C residue, meanwhile MspI activity is blocked only by methylation at the external C.

B. Thirty ug genomic DNAs extracted from GFP, GxA, P69c x (GxA) and *sde1* lines were digested with HaeIII, respectively. Hybridization probe was derived from the full length *GFP* gene. The sizes of hybridized DNA fragments were indicated by arrows at the side. Lower panel shows the diagram of HaeIII cleavage sites within GFP transgene.

efficient (**Figure 4.7 A**, top right panel). This pattern of demethylation was similar to that found previously in the *sgs2* mutant in the L1 background (**Figure 4.7 A**, middle panel) (Mourrain et al., 2000). Thus, p69 expression also resulted in the demethylation of *GUS* DNA as completely as in the *sgs2* mutant.

As shown in the previous section, the accumulation of *PVX-GFP* and *GFP* siRNA was increased in (GxA) x P69c plants. It was very interesting to know whether demethylatiom also happened in (GxA) x P69c plants. The results showed that, while partially resistant in GxA plants, the *GFP* DNA was completely digested by *HaeIII* in (GxA) x P69c as in G and the *sde1* (GxA) mutant (**Figure 4.7 B**). Interestingly, enhanced accumulation of viral siRNAs and loss of the *GFP* DNA methylation were both observed in the *sde1*(GxA) mutant (Dalmay et al., 2000b).

4.3.6 p69 does not inhibit PTGS induced by IR-RNA transgenes

RNA silencing induced by IR-RNA transgenes is distinct from sense and virus RNA silencing as neither the cellular nor viral RdRP is involved in the formation of the dsRNA trigger from a nuclear self-complementary IR-RNA transcript (Beclin et al., 2002). A current model to explain PTGS induced by sense transgene (S-PTGS) in higher plants is that transcription of the transgene produces aberrant RNA because of methylation and chromatin structural changes occurring in the locus of the transgene. Aberrant transcripts form a local duplex structure that is used as substrate by SDE1/SGS2 - a plant specific RdRP for dsRNA synthesis, which serves as the iniator of PTGS (Vance and Vaucheret, 2001; Vaucheret et al., 2001; Hannon, 2002). However, dsRNA is formed after transcription of an IR-RNA transgene, thus IR-PTGS is independent of *SDE1/SGS2* (Beclin

et al., 2002). This model is supported by genetic analysis. For example, expression of the silenced *GUS* sense transgene in L1 line is restored in *sds1/sgs2* mutants (Mourrain et al., 2000), whereas IR-PTGS is independent of *SGS2/SDE1*, *SGS3*, or *AGO1* (Beclin et al., 2002d). The latter report also showed that cucumber mosaic cucumovirus (CMV), turnip crinkle carmovirus (TCV), turnip mosaic potyvirus (TuMV), and turnip vein clearing tobamovirus (TVCV) were all able to suppress both S-PTGS and IR-PTGS (Beclin et al., 2002). The likely explanation is that these viruses encode factors that act downstream of the complex of AGO1, RdRP (SGS2/SDE1), SGS3 and SDE3 in dsRNA synthesis in suppressing PTGS.

To determine if p69 inhibits IR-PTGS, P69c was crossed with a transgenic line (*IRRbx1*) containing a construct producing IR-RNA targeting *RING BOX 1* (*Rbx1*) gene. As mentioned above, in *Arabidopsis*, an endogenous gene could be silenced by a homologous IR-RNA transgene (Chuang and Meyerowitz, 2000; Smith et al., 2000). *Rbx1* gene is a key factor in the ubiquitination pathway for protein degradation. Because loss of function of *Rbx1* is lethal (Xu et al., 2002), the expression of *IRRbx1* in IRRbx1 line was engineered to be under the control of an inducible promoter GVG. IRRbx1 plants display the lethal phenotype only when plants are grown in the medium containing DEX. The *IRRbx1/-* heterozygote was crossed with P69c and C24, respectively, and the F1 progenies were allowed to grow in the MS medium, MS plus hygromycin (for selection of *IRRbx1* and /or *p69* positive individuals), and MS plus DEX (for the transcriptional induction of *IRRbx1*), respectively. The progeny of *IRRbx1/-* self-pollinated as well as P69c and C24 WT were used as controls. Under hygromycin selection, all genotypes showed the expected segregation ratio of hygromycin sensitive (HYG^S) to resistant (HYG^R) (**Table**

Table 4.2

Line	HYG^R	HYG^S	DEX^R	DEX^S
IRRbx1/-, self	26.5	9.0	10.25	31.75
Wt x IRRbx1/-	20.75	21.0	18.75	20.25
P69c x IRRbx1/-	45.5	0	17.7	19.3
Wt	0	47.0	53.3	0
P69c	49.5	0	42.2	0

Average data were obtained from three independent experiments.

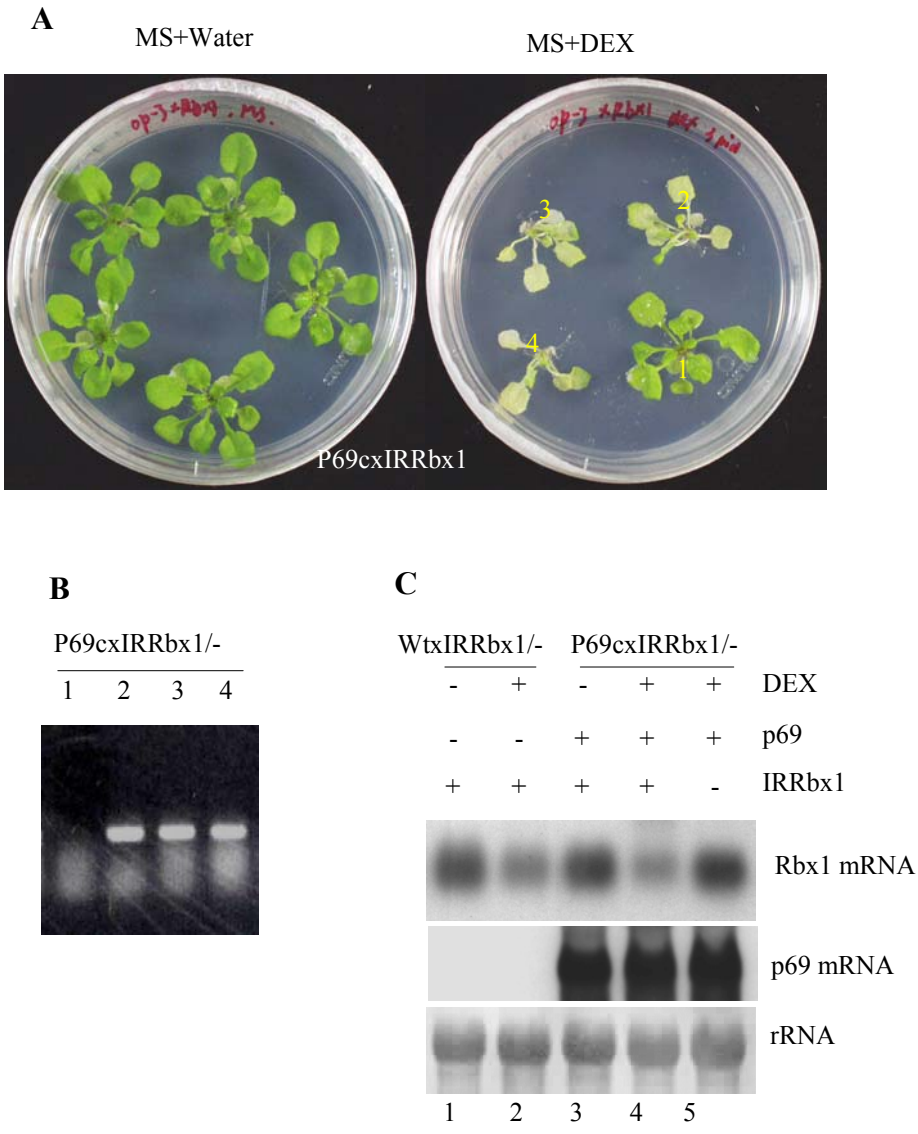


Figure 4.8

Table 4.2. IRRbx1 (linked with hygromycin resistance) heterozygote was cross-pollinated with P69c (linked with hygromycin resistance) homozygote and C24, respectively. F1 seeds were allowed to germinate and grow on a) MS medium alone; b) MS plus 20μg/ml hygromycin; and c) MS plus 0.2 μM DEX (catalog No. D-1756, Sigma). . Meanwhile, the progeny of *IRRbx1*/+ self-pollinated together with P69c and C-24 WT were used as controls. Germination rates of all seeds on MS were 100% (data not show). The average numbers of resistant and susceptible plants on hygromycin and DEX medium are given (Table 4.2). Numbers of plants were the average from three independent experiments. F1 from the selfed IRRbx1 heterozygote displayed the expected DEXS/DEXR ratio of 3:1; however, F1 from both Wt x IRRbx1/- and P69c x IRRbx1/- showed 1:1 segregation ratio.

Figure 4.8 p69 does not suppress PTGS induced by IR-RNA transgenes.

A. F1 seedlings of the P69c x IRRbx1 cross grown to 6-7 leaves in MS medium were transferred to MS plates with or without DEX and grown for anadditional three days. Plate on left: representatives not treated with DEX. Individuals were indistinguishable from each other and all resembled the P69c control. Plate on right: representatives treated with DEX. Unlike DEX^R individuals, DEX^S individuals were smaller and displayed pale yellow leaves.

B. Before DEX treatment, one leaf was taken from each plant separately to extract DNA. To detect IRRbx1 transgene, PCR method was used to amplify a fragment of IRRbx1 (see Material and method). A total of 16 plants was genotyped before DEX treatment. 7 plants had the IRRbx1 insertion, all these 7 plants showed IRRbx1 phenotype, meanwhile 8 plants without the IRRbx1 insertion showed normal phenotype. Genotyping by PCR showed that all of the three DEX^S individuals contained the IRRbx1 transgene (lanes 2-4) whereas the DEX^R individual did not (lane 1). Since all contained the *P69* transgene, this result indicates that P69 had no effect on the DEX^S phenotype.

C. p69 did not prevent the DEX-dependent knockdown of the endogenous *Rbx1* mRNA. Total RNA extracted from plants of various genotypes indicated above the lanes was analyzed for the accumulation of the *p69* or *Rbx1* mRNA. It was noted that presence of the *p69* transgene consistently caused further reduction (>20%) of the *Rbx1* mRNA after DEX induction of the IRRbx1 transgene (compare lanes 2 and 4).

4.2). Both P69c and C24 plants were insensitive to DEX (**Table 4.2**). In addition, the progenies of *IRRbx1/-* self-pollinated and C24 x *IRRbx1/-* displayed the expected segregation ratio of DEX^R:DEX^S of 1:3 (10.25/31.75) and 1:1 (18.75:20.25), respectively (**Table 4.2**). All F1 plants of P69c x *IRRbx1/-* would be DEX resistant if p69 suppresses IR-PTGS because all F1 individuals carry *p69* gene. On the other hand, if p69 does not suppress IR-PTGS, a 1:1 segregation ratio of DEX^R:DEX^S should be observed because only half of the F1 individuals carry *IRRbx1* transgene. In fact, a segregation ratio of DEX^R:DEX^S close to 1:1 (17.7:19.3) was observed for the progeny of P69-3 x *IRRbx1/-* grown in the DEX medium (**Table 4.2**).

To determine their genotypes, F1 seedlings from the P69c x *IRRbx1*/- cross were grown to 6-7 leaves in hygromycin medium before they were transferred to MS plates with or without DEX and grown for additional three days (**Figure 4.8 A**). Genotyping by PCR (**Figure 4.8 B**) showed that among the F1 plants from P69c x *IRRbx1*/- that carried *35S-P69*, *IRRbx1* was detected in the three DEX^S individuals (**Figure 4.8 A**, plant 2, 3, 4) but not in the DEX^R individual (**Figure 4.8 A**, plant 1). Consistent with the phenotypic analysis, p69 expression was not associated with an increased accumulation of the *AtRbx1* mRNA (**Figure 4.8 C**, compare lanes 2 and 4). Surprisingly, there was in fact an obvious decrease in the accumulation of *AtRbx1* mRNA in the P69C x *IRRbx*1 plants after DEX treatment, suggesting an intriguing possibility that P69 may enhance RNA silencing mediated by IR-RNA. Thus, the RNAi phenotype was determined solely by the IR-RNA transgene and it was not suppressed by the presence of *35S-P69*. Potent p69 suppression of RNA silencing triggered by sense-RNA transgenes but not by IR-RNA transgenes indicates that p69 may disrupt a cellular function leading to dsRNA synthesis.

As an independent confirmation, I made an IR-RNA construct carrying *IRPDS* driven by 35S promoter and this construct was transformed to Col-0 Wt plants. *PDS* gene encodes a phytoene desaturase which is involved in chlorophyll synthesis. If *PDS* is knocked out, the plant will show photo-bleaching phenotype under intensive light. Five transformants were obtained. One of them (**Figure 4.9 A**, left plant) was completely white and did not produce any seeds. The rest of the transformants were completely white at the cotyledon stage, but their true leaves were green and white mosaic as shown in **Figure 4.9 A** (middle and right plants). Three of these lines with photo bleaching phenotype were found to carry a single copy of transgene, according to a hygromycin resistance segregation ratio at 3:1 among their selfed progenies.

An *IRPDS/-* heterozygote (IRPDS-2) that contained a single copy of the transgene was crossed with P69c (in C24 background) and C24, respectively, and the F1 progenies were allowed to grow in the MS medium and MS plus hygromycin (for selection of the *IRPDS* and/or *35S-P69* positive individuals). The progeny of C24x*IRPDS/-* segregated 1:1 of hygromycin sensitive (HYG^S) to resistant (HYG^R), but all of the 12 HYG^R individuals showed photo-bleaching (**Figure 4.9 C**). All of the 19 progeny from P69c x *IRPDS/-* carried the *35S-P69* transgene and were HYG^R; however, only 10 of these F1 plants showed photo-bleaching (**Figure 4.9 D**). When grown on MS medium (**Figure 4.9 B**), both F1 populations from crosses C24x*IRPDS*/- (n=29) and P69CxIRPDS/- (n=52) contained approximately 50% individuals displaying the photo-bleaching phenotype. These results indicate that the presence of *35S-P69* did not suppress the *PDS* silencing phenotype mediated by *IRPDS*, providing an independent confirmation that p69 suppression of PTGS may occur upstream of dsRNA synthesis.

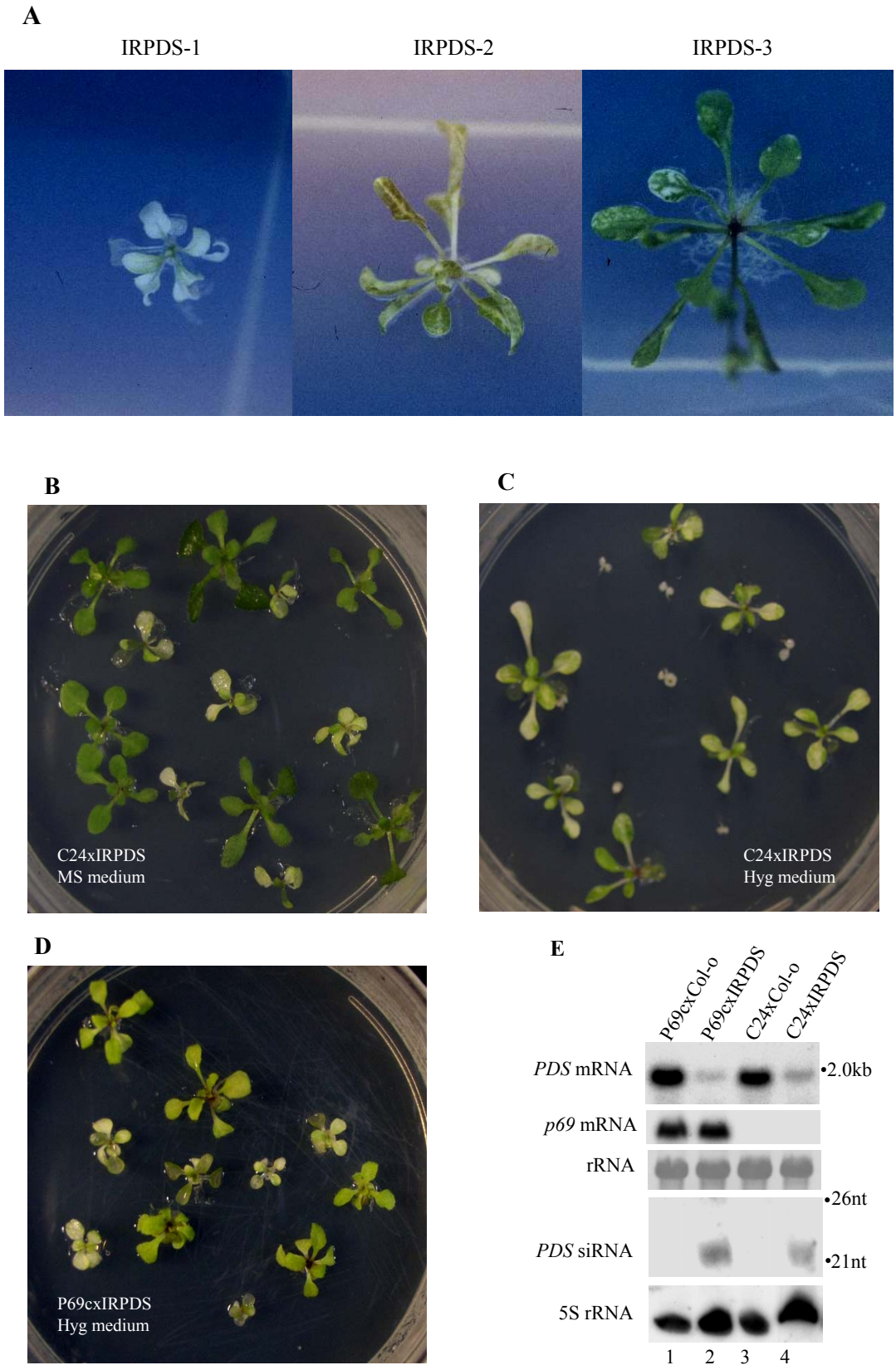

A
IRPDS-1
IRPDS-2
IRPDS-3
B
C24xIRPDS
MS medium
C
C24xIRPDS
Hyg medium
D
P69cxIRPDS
Hyg medium
E
P69cxCol-o
P69cxIRPDS
C24xCol-o
C24xIRPDS
PDS mRNA
•2.0kb
p69 mRNA
rRNA
•26nt
PDS siRNA
•21nt
5S rRNA
1 2 3 4

Figure 4.9

Figure 4.9 p69 does not suppress PTGS of endogenous *PDS* gene induced by *IRPDS* transgene.

A. Three *IRPDS* silencing lines showed the photo-bleaching RNAi phenotype.

B-D. Genetic segregation of the photo-bleaching phenotype of the *IRPDS* transgene. *IRPDS-2* heterozygote (*IRPDS*/-) was crossed with either C-24 or an P69c homozygote and the F1 progenies grown in MS or MS+hygromycin (Hyg) media. A representative plate is shown for each except for P69c x IRPDS that did not show difference with or without Hyg selection. Note the difference between P69c plants and photo-bleached plants (C).

E. Detection of *p69* mRNA and *PDS* mRNA/siRNAs. Individual F1 plants were pooled according to their genotypes for extraction of high and low molecular weight RNAs. Equal loading was monitored for mRNA by rRNA staining and for small RNAs by stripping and re-probing the same filter for 5S rRNA. The positions of 21- and 26-nt RNA markers are indicated.

RNA analysis showed that expression of the *IRPDS* transgene in C24x*IRPDS* plants resulted in the accumulation of *PDS*-specific siRNAs (lane 4, **Figure 4.9 E**) and an approximately 75% knockdown of the *PDS* mRNA (compare lanes 3 and 4, top panel of **Figure 4.9 E**). Consistent with lack of p69 suppression indicated by the phenotypic analysis, p69 expression in the *IRPDS*-expressing plants was not associated with an increased accumulation of *PDS* mRNA (compare lanes 2 and 4, **Figure 4.9 E**). Surprisingly, there was in fact an approximately 60% increase in the accumulation of *PDS* siRNAs and a corresponding decrease in the accumulation of *PDS* mRNA associated with P69 expression (**Figure 4.9 E**, compare lanes 2 and 4). This result suggests an intriguing possibility that P69 may enhance RNA silencing mediated by IR-RNA.

As a further confirmation, another IR-RNA (*35S-IRGFP*) transgene targeting *35S GFP* was introduced into line G that carries a single copy of 35S-GFP (Dalmay et al., 2000b). 22 resulting transformants lines that were red fluorescent under UV were obtained, 7 of which contained a single copy of the *35S-IRGFP* transgene according to a hygromycin resistance segregation ratio at 3:1 among their selfed progenies. All hygromycin resistant progenies were red fluorescent, indicating that the silencing *IRGFP* transgene was dominant as expected (**Figure 4.10 A**, right plant). Analyses of mRNA and siRNA in the progeny plants showed that *35S-GFP* was effectively silenced by the *IR-RNA* transgene (**Figure 4.10 C**, compare lanes 1 and 2). Three such lines containing a single *35S-IRGFP* transgene were selected for TYMV infection. Systemically infected GFP x IRGFP plants exhibited typical TYMV symptoms, but *35S-GFP* remained silenced (**Figure 4.10 B**, left plant). Northern blot analysis revealed that p69 expression was again associated with a further decrease in the accumulation of *GFP* mRNA (**Figure 4.10 C**, upper panel, compare lanes 2 and 3). The accumulation of *GFP* siRNAs in these plants

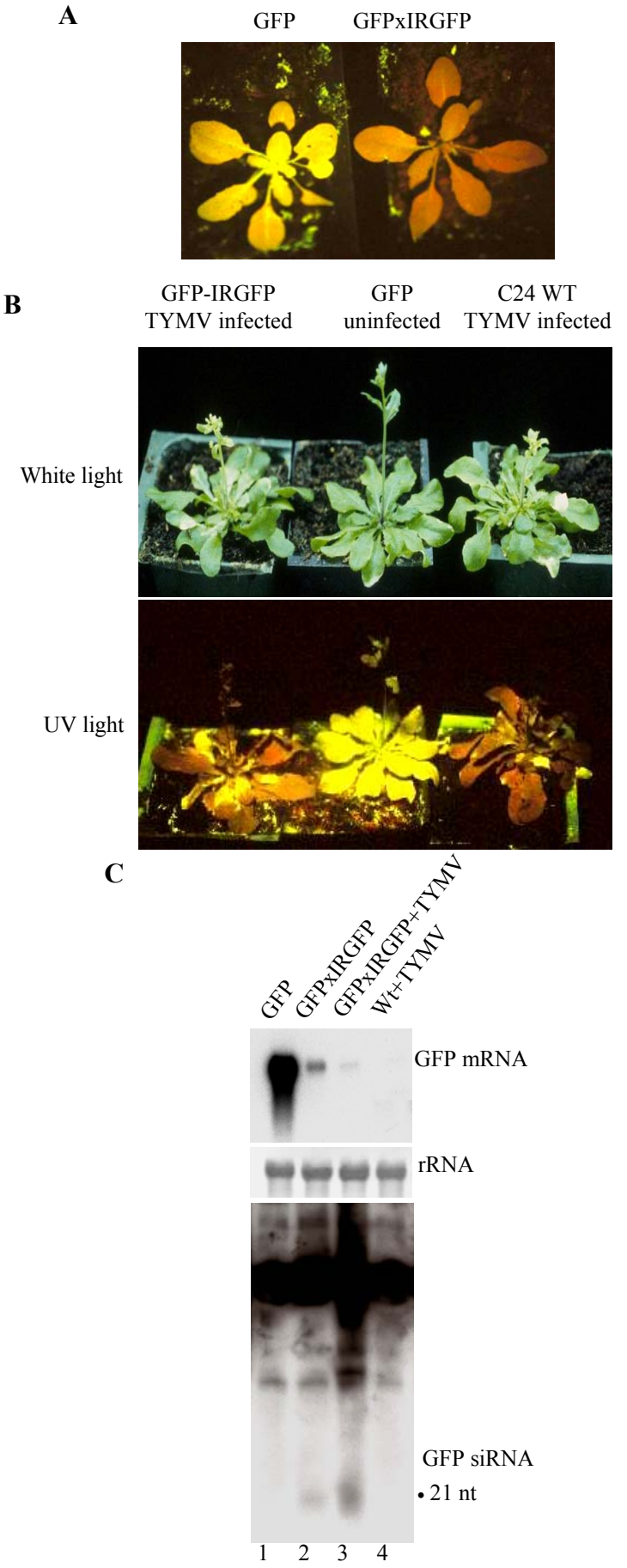

A
GFP
GFPxIRGFP
B
GFP-IRGFP
TYMV infected
GFP
uninfected
C24 WT
TYMV infected
White light
UV light
C
GFP
GFPxIRGFP
GFPxIRGFP+TYMV
Wt+TYMV
GFP mRNA
rRNA
GFP siRNA
• 21 nt
1 2 3 4

Figure 4.10

Figure 4.10 TYMV does not inhibit RNA silencing induced by *IRGFP* transgenes.

A. GFP x IRGFP silencing plant (right) and 35S-GFP expression plant (left) under UV light.

B. TYMV does not inhibit GFP silencing induced by *IRGFP*. *GFP* expression line G was transformed with IRGFP construct. Plants with 6-8 leaves from a single locus *IRGFP* insertion line were inoculated with TYMV. Plant on the left is GFP x IRGFP silencing plant infected with TYMV at 20 dpi, in which systemic leaves of all infected plants remained red under UV light through whole life (lower panel: left plant). Plant in the middle is GFP expression plant. Plant on the right is C24 Wt plant infected with TYMV at 20 dpi. White patches under UV light from C24 and GFPxIRGFP plants were due to the necrosis caused by TYMV infection. Top panel is under white light. Lower panel is under UV light.

C. Top panel: TYMV inoculation did not restore the expression of *GFP* transgene silenced by *IRGFP*. Five ug total RNA was loaded. Bottom panel: TYMV did not bring down the accumulation of *GFP* siRNA in IRGFP induced GFP silencing. Twenty ug siRNA was loaded. Systemic leaves at 20 dpi were harvested for the RNA extraction.

was further analyzed. In agreement with a decreased accumulation of GFP mRNA, a significant increase was detected in the accumulation of GFP siRNAs following TYMV infection (**Figure 4.10 C**, lower panel, compare lanes 2 and 3). These results illustrate that P69 expressed from TYMV also did not inhibit silencing of *35S-GFP* mediated by the IR-RNA transgene and in fact was correlated with a positive effect on IR-RNA-induced silencing, which further confirmed the results in P69c x IRPDS experiment.

4.4 Discussion

4.4.1 TYMV p69 is a suppressor of PTGS

A number of independent assays were used to determine if p69 is a suppressor of RNA silencing as suggested in Chapter 3. First, the *Agrobacterium* infiltration system was used to demonstrate that TYMV p69 acted as a suppressor of PTGS although its activity is weak and transient as compared to CMV 2b in *N. benthamiana*. Second, using a recombinant TRV as a vector to deliver p69 to the *Arabidopsis* silencing plants confirmed that p69 was an active suppressor of RNA silencing in *Arabidopsis* plants. Third, by a genetic approach, p69 transgene strongly inhibits PTGS either induced by sense-RNA transgenes or by a virus-derived amplicon transgene in *Arabidopsis.* All these results prove our prediction that p69 is indeed a suppressor of RNA silencing.

Suppression of sense-RNA silencing by p69 was found to be weaker in *N. benthamiana* than in *Arabidopsis*, because siRNA accumulation was detectable and gradually increased with time during after GFP co-infiltration with *p69* on *N. benthamiana* leaves, whilst siRNA was undetectable in P69c x L1 *Arabidopsis* plants. Systemic silencing was only delayed in p69 and GFP co-infiltrated plants compared to that infiltrated with GFP alone. These results suggest that TYMV p69 may not be adapted to

function in a non-host species and that the p69-analogue encoded by *Solanaceae*-specific tymovirus such as Eggplant mosaic virus (EMV) may act as a stronger PTGS suppressor in *N. benthamiana* than p69 of TYMV.

4.4.2 p69 suppresses PTGS at the upstream of dsRNA synthesis

Suppression activity of p69 in *Arabidopsis* exhibits a number of unique features. Firstly, p69 suppression of PTGS mediated by the sense-RNA *GUS* transgene is as complete (**Figure 4.4**) as in the *sgs2* mutant that contains a genetic mutation in the *SGS2/SDE1* locus encoding a cellular RdRP (Mourrain et al., 2000). In the p69-expressing L1 plants, the expression level of the *GUS* transgene was very high, the *GUS* siRNAs were undetectable and the *GUS* DNA was mostly demethylated (**Figure 4.7**). This is in contrast to HC-Pro suppression of PTGS targeted against a similar *GUS* transgene in tobacco, which was not associated with any detectable decrease in transgene methylation although there were high levels of the *GUS* transgene expression and the elimination of siRNAs (Mallory et al., 2001). Similarly, transgenic expression of the cucumber mosaic virus 2b protein significantly reduced methylation of the *GUS* transgene, but it did not eliminate siRNA production (Guo and Ding, 2002).

Several lines of evidence also indicate that among the viral suppressors characterized to date p69 targets a unique step in the RNA silencing pathway. The fact that p69 suppressed PTGS induced by sense-RNA transgenes but not by IR-RNA transgenes indicates that p69 inhibits a cellular function leading to dsRNA production. This conclusion is consistent with the observation that p69 had similar effects on RNA silencing as genetic mutations in cellular genes involved in dsRNA production such as *SGS2/SDE1*. For example, both p69 expression and the *sgs2/sde1* mutation eliminated

siRNA production (**Figure 4.4**) and DNA methylation (**Figure 4.7**) associated with the autonomously silencing *GUS* transgene at the L1 locus.

In the amplicon-based two-component silencing system, both *p69* expression and the *sgs2/sde1* mutation suppressed RNA silencing and DNA methylation of the *35S-GFP* transgene (**Figure 4.5, 4.7**), but both increased the accumulation of virus-specific siRNAs (Dalmay et al., 2000e). This result further indicates that p69 may prevent dsRNA synthesis by the cellular RdRP pathway but not by the viral RdRP. The differences between p69 expressing and *sgs2/sde1* mutant plants, such as PVX-GFP RNAs was much higher and GFP mRNA was lower in p69 x (GxA) plants than in *sgs2/sde1* mutants, were perhaps due to p69 suppressing not only *SGS2/SDE1* cellular RdRP function, but also the function of other members of 4-member RdRP family in *Arabidopsis* (Dalmay et al., 2000). *SGS2/SDE1* has no general role in antiviral defense. A recent study has shown that *AtRdRP1*-another *Arabidopsis* RdRP plays an important role in antiviral defense. *AtRdRP1* is induced by salicylic acid treatment and virus infection (Yu et al., 2003). The *AtRdRP1* knockout mutant accumulated higher and more persistent levels of viral RNAs in both the lower, inoculated and upper, systemically infected leaves than did wild-type plants, when infected by a tobamovirus and a tobravirus. These results indicate that SGS2/SDE1 protein only recognize aberrant mRNAs transcribed from methylated regions and AtRdRP1 does not recognize aberrant mRNAs rather than aberrant viral RNAs. p69 may block both of these two RdRP functions to produce greater effect on PVX-GFP replication, then PVX-GFP RNAs and siRNAs accumulation are all increased in p69 expressing GxA plants. A similar role was proposed previously for the 25K protein (p25) encoded by PVX (Voinnet et al., 2000). Interestingly, both p25 (Voinnet et al., 2000) and p69 (Bozarth et al., 1992) are essential for virus cell-to-cell movement in contrast to a

dispensable role for other viral suppressors in the cell-to-cell movement. Unlike p69, however, p25 is not active in the suppression of either established silencing targeted against sense-RNA transgenes (Brigneti et al., 1998; Voinnet et al., 1999) or silencing targeted against its own amplicon transgene (Angell and Baulcombe, 1997; Dalmay et al., 2000a). Furthermore, it appears that p25 expression also does not prevent DNA methylation of the *35S-GUS* transgene in the tobacco host (Mallory, 2003). A recent work suggests that p25 acts downstream of dsRNA by a specific inhibition of the production of the longer siRNA species of 24-26 nt (Hamilton et al., 2002). The NSs protein encoded by tomato spotted wilt virus also suppresses sense RNA-induced PTGS but not PTGS triggered by an IR-RNA transgene, as shown in the *Agrobacterium* co-infiltration experiments (Takeda et al., 2002), and further characterization will be necessary to determine if NSs is mechanistically similar to p69.

Chapter 5

p69 upregulates the role of miRNAs in the negative control of host gene expression

5.1 Introduction

How virus infection impacts biochemically and physiologically upon its hosts at the level of cells, tissues and whole plants remains one of the gaps in our knowledge in plant virology. After the first infection event, which occurs after mechanical damage to the cell wall and plasma membrane, the virus remains within the symplast (Carrington and Whitham, 1998). Distant cells are infected only after passage through plasmodesmata. In the absence of an active resistance response (e.g. the hypersensitive response), the cells that have supported a complete infection "cycle" do not die but retain large quantities of virus, while the infection moves to adjacent tissues. Finally, the outcome of the initial infection event and the progressive spread of the virus to most or all susceptible tissues, is the appearance of symptoms. These symptoms represent the consequence of physiological and structural changes at the cellular level and alterations in physiology that often are associated with the reduced growth and development of the whole plant, which can range from mild discoloration to severe developmental defects and death (Mathews, 1991). The fact that most symptoms occur in new tissues arising after initial infection suggests that disease involves disruption of normal growth and development. But the molecular mechanism of viral disease symptom development is not clear yet.

RNA silencing is part of a larger set of pathways involving small RNAs (Hannon, 2002). Developmental regulation in plants and animals requires miRNAs, which are

present in one polarity and are derived from RNA transcripts that have potential to form hairpin structures (Pasquinelli and Ruvkun, 2002). Processing of both miRNA and siRNA precursors in animals requires *DICER* (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001). In plants, the multidomain DCL1 catalyzes miRNA precursor processing (Reinhart et al., 2002; Park et al., 2002a), although its role in siRNA generation is not established. Biochemical data indicate that different subclasses of silencing-associated small RNAs require different DICER-like proteins in plants (Tang et al., 2003; Xie et al., 2003; 2004). The *dcl1* mutants exhibit a range of embryo, vegetative, and meristem defects (Schauer et al., 2002), likely because they contain low levels of miRNAs.

miRNAs appear to negatively regulate genes required for stem/meristem cell identity, developmental timing, and other developmental processes by interacting with mRNAs encoding key regulatory factors (Rhoades et al., 2002; Pasquinelli et al., 2000; Ambros et al., 2003). Interaction between an miRNA and an mRNA target can have at least two consequences. First, as shown for the small temporal RNAs of *C. elegans*, interaction through imperfect base pairing with the 3' nontranslated region of the target can lead to translation arrest (Olsen and Ambros, 1999; Wightman et al., 1993; Chen, 2003). Second, as shown for miR171 from *Arabidopsis*, perfect base pair interaction can trigger mRNA cleavage by a mechanism that resembles siRNA-guided cleavage (Llave et al., 2002). A translation arrest-type miRNA can be converted to a target degradation-type miRNA by increasing the degree of complementarity between miRNA and target sequences (Hutvagner and Zamore, 2002). Recent research showed that miRNAs with 1-3 nt mismatches to their targets also can trigger cleavage on their target mRNAs (Kasschau et al., 2003). Fourteen out of 16 *Arabidopsis* miRNAs were predicted to be

complementary to 49 identifying mRNAs, most of them are transcriptional factors (Rhoades et al., 2002).

In Chapter 4, it was demonstrated that p69 suppression of RNA silencing occurs upstream of dsRNA. Since p69-expressing plants also exhibited disease symptom-like developmental abnormalities (see below), I investigated whether p69 interfered with the miRNA pathway in *Arabidopsis*. These analyses revealed that p69 expression resulted in the induction of the miRNA pathway, leading to elevated miRNA levels and reduced accumulation of four cellular mRNAs targeted for cleavage by these miRNAs. Further analyses showed that p69 expression was associated with transcriptional induction of both *DCL1* and *SDE1/SGS2*, suggesting that the observed up-regulation of the miRNA pathway is a result of a negative feedback regulation of the RNA silencing pathway by p69 suppression. The possibility that miRNAs can play a pathogenic role in the induction of viral diseases is discussed.

5.2 Materials and methods

5.2.1 Materials

Arabidopsis lines used in this study were G, P69c, P69d and L1 (GUS silencing line), *sgs2* mutant and Wt C24.

5.2.2 RNA Analysis

Analysis of high and low molecular weight RNAs was performed as described in Section 3.2.2. Total RNA of newly bolted inflorescences of plants from C24, L1, *sgs2*, P69c, P69d, G and C24 systemically infected with TYMV and from C24, P69c and P69d 6-day-old seedling grown on filter paper and from leaves and stems was isolated. For high molecular weight Northerns, hybridization probes were labeled with ^{32}P using the

Amersham Megaprime DNA labeling kit. *SPL3*(At2g33810), *SCL6-IV*(At4g00150), *SPL2*(At5g43270) and *SPL9*(At2g42200) probes corresponded to nucleotides 293-532, 774-1485, 723-1019 and 207-563 of the *SPL3*, *SCL6-IV*, *SPL2 and SPL9* mRNAs, respectively. Northern blots detecting low molecular weight RNA, DNA oligonucleotides complementary to miRNA were end-labeled with ^{32}P as probes using T4 polynucleotide kinase (Roche Applied Science).

5.2.3 RLM-RACE

The FirstChoice™ RLM-RACE Kit (Ambion) was used following manufacturer's instructions. Briefly, total RNAs were extracted from flowering P69C plants and poly(A)$^+$ mRNA was obtained by two rounds of purification with an Oligotex mRNA Midi Kit (Qiagen). The RNA was directly ligated to RLM-RACE 5'RACE RNA Oligo adaptor (45 nt). The oligo dT(15 mer) primer was used to prime cDNA synthesis with reverse transcriptase. The resultant cDNA was used for the first round nested PCR using the 5'RACE Outer Primer together with a gene-specific outer primer, which was complementary to nucleotides 690-710 of *SPL3* (At2g33810 complete cDNA), 1166-1185 of *SPL2* (At5g43270), or 1075-1094 of *CUC2* (At5g53950). The 5'RACE inner Primer were used in the second round nested PCR with a second set of gene-specific Inner Primers, respectively complementary to nucleotides 665-685 of *SPL3*, 1134-1152 OF *SPL2*, and 1057-1078 of *CUC2*. In each case, a unique gene-specific DNA fragment was amplified (**Figure 5.3 A**). The PCR products were gel purified and cloned into pGEM-T Easy vector (Promega) for DNA sequencing.

5.2.4 Real Time RT-PCR Analysis

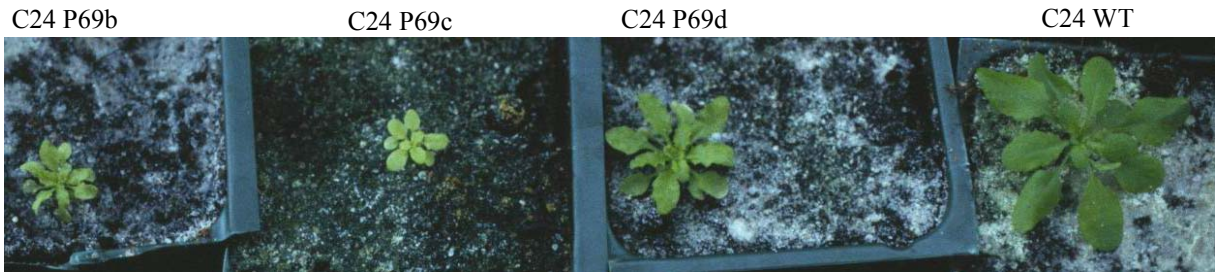
Total cDNA was first synthesized using the oligo-dT15 primer from the total RNA extracted from whole plants without roots and used as templates for the real time PCR with gene-specific primers. The amplified regions corresponded to nucleotides 721-949, 628-1057, 484-748, and 500-992 of the *SDE1/SGS2* (*RdRP*), *DCL1*, *AGO1* and *β-TUBULIN-1* mRNAs, respectively. The LightCycler-FastStartDNA Master SYBR Green Kit and LightCycler Instrument Real time PCR machine (Roche Applied Science) were used. The PCR reactions were stopped when PCR was in the log-linear phase before the later plateau phase, at 27 cycles for *RdRP* and 29 cycles for *DCL1*, *AGO1*, and *β-TUBULIN-1*. After confirming the identity by direct sequencing, PCR products were blotted to a nylon membrane and detected by hybridizations with gene-specific probes.

5.3 Results

5.3.1 p69 transgene causes severe disease symptoms in transgenic *Arabidopsis* plants

As described in Section 4.3.3, *p69* gene was introduced into *Arabidopsis* plants for genetic studies (**Figure 4.1 A**). It is striking that 7 of the 11 *35S-P69* transformants obtained showed disease symptom-like abnormal plant development (**Figure 5.1 A**, plant 1, 2, and 3; **Figure 5.2 A**, left plant). The 35S-ΔP69 gene was also transformed to C24. All 8 35S-ΔP69 transformants obtained showed a normal phenotype. The abnormal development co-segregated with the transgene in backcrosses with Wt plants. Characteristics of the abnormal development included severe dwarf, pale-colored leaves with significantly reduced cell sizes, late flowering, short siliques and decreased fertility.

A


C24 P69b
C24 P69c
C24 P69d
C24 WT

B

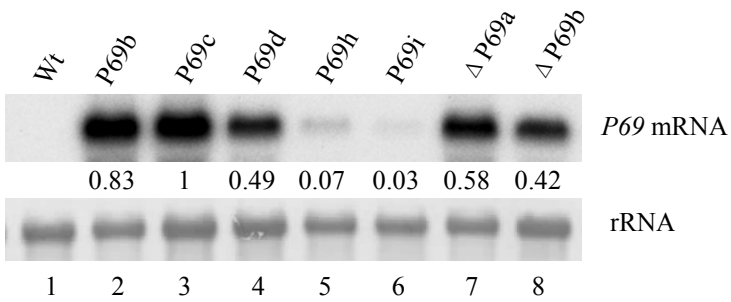

Wt
P69b
P69c
P69d
P69h
P69i
Δ P69a
Δ P69b
P69 mRNA
0.83
1
0.49
0.07
0.03
0.58
0.42
rRNA
1
2
3
4
5
6
7
8

Figure 5.1

Figure 5.1 TYMV *p69* transgenic plants with high levels of p69 mRNA displayed a severe disease phenotype.

A. In total, 7 out of 11 transformants displayed a sever disease phenotype as shown. Top panel: rosette stage. Three independent *P69* transgenic lines, each showing 3:1 segregation ratio on hygromycin medium after selfing, are shown. Among the three lines, P69c has the strongest phenotype, followed by P69b and then the lowest P69d.

B. The severity of phenotype was correlated to the level of *p69* expression. P69h, P69i (two of four similar P69 transgenic lines) and ΔP69a, ΔP69b (two of eight ΔP69 transgenic lines) showed no disease symptom phenotypes. The RA level for each samples is indicatd (the p69 level in P69c plants was arbitrarily designated as 1.0). Three ug total RNA was loaded in each lane. Methylene blue staining Of RNA was used as a loading control.

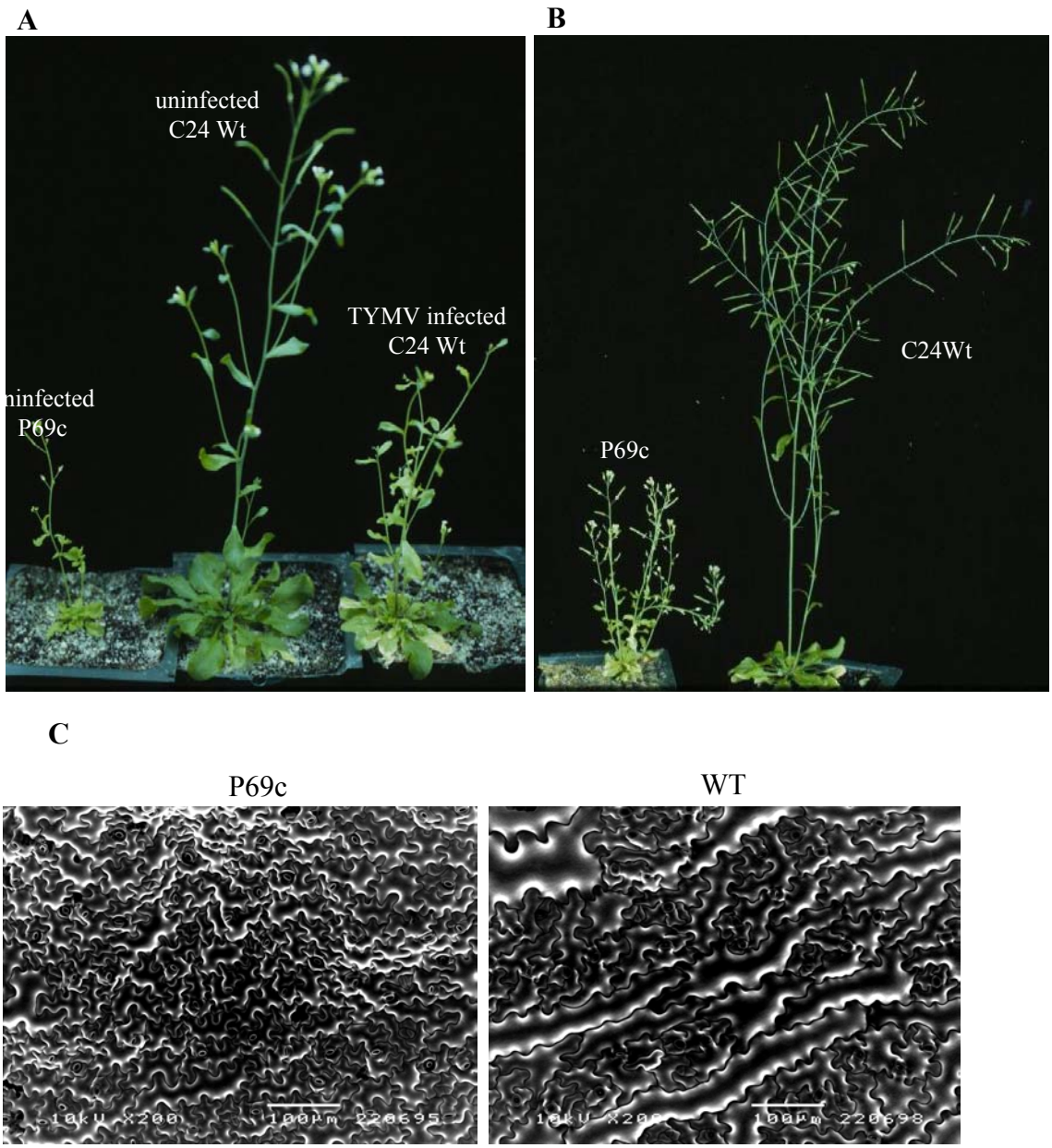


Figure 5.2

Table 5. 1

	Days from sowing to the first flowering	Rosette leaves at the first flowering
WT	42.4	11.8
P69c	48.5	13.4

Figure 5.2 P69c transgenic line.

A. Transgenic line P69c (plant on the left) expressing *p69* causes pleiotropic developmental defects in *Arabidopsis* that resembles the phenotype displayed by a plant infected systemically with TYMV (plant on the right). A Wt control plant is shown in the middle.

B. P69c plant showed decreasing fertility (left plants). A Wt control plant is shown on the right.

C. Epidermal cells of leaves in P69c (left) were much smaller than those in Wt (right). The SEM images were taken from the abaxial surface of fully expanded leaves.
Cell number per area of leaf abaxial in P69c plant (left) was twice that in Wt (right).

Table 5.1 P69 plants are late flowering. The data for P69c were on the average of 65 P69c plants. The data for Wt were on the average of 61 Wt plants.

Three independent *p69* transgenic lines with single locus insertion (each showing 3:1 segregation ratio on hygromycin medium after selfing) are shown in **Figure 5.1 A**. Among the three lines, p69c has the strongest phenotype, followed by p69b and then the lowest p69d. The severity of phenotype was correlated to the level of *p69* expression (**Figure 5.1 B**). The expression of *p69* RNA was hardly detectable in the remaining 4 lines (**Figure 5.1 B**, lanes 5, 6 showed two lines P69h, P69i.) that were morphologically indistinguishable from either Wt plants or any of the 8 *35S-ΔP69* transformants. *p69* mRNA in 35S-ΔP69 transformants was also presented very high levels (**Figure 5.1 B**, lanes 7, 8). Line P69c was used for further investigation. **Table 5.1** shows that the flowering time was delayed in P69c plants. P69c homozygous plants began flowering at 48.5 days after sowing with 13.4 rosette leaves on average (n=65) in contrast to Wt plants that flowered at 42.4 days after sowing with 11.8 rosette leaves (n=61) under the growth conditions as described in section 2.1. As the SEM images showed (**Figure 5.2 C**), the cell sizes of leaves in P69c plants were much smaller than those in Wt. Perhaps this is why P69 plants are much smaller than the Wt plants. The developmental defects observed in the P69c plants mimicked, but were more severe than, the disease symptoms found in Wt plants systemically infected with TYMV (**Figure 5.2 A**, right plant). **Figure 5.2 B** showed that fertility and silique size were reduced in P69c plant (left) compared to Wt (right). Thus, p69 alone is able to confer virulence independent of TYMV infection, further confirming the virulence function of *p69* (Tsai and Dreher, 1993; Bozarth et al., 1992).

5.3.2 p69 expression enhances miRNA accumulation

To investigate if the developmental defects associated with p69 expression are due to an altered miRNA pathway, total RNA was extracted from newly bolted inflorescence of P69c plants and analyzed for the accumulation of seven *Arabdopsis* miRNAs. Among these five (miR156, miR162, miR164, miR167 and miR171) are conserved in rice (a monocotyledonous species) and two (miR157 and miR158) may be specific to *Arabidopsis* (Rhoades et al., 2002). It was found that each of the seven miRNA increased in abundance in P69c plants (**Figure 5.3 A**, left panel; **Figure 5.3 B**). To further demonstrate that the increase of miRNA accumulation was not caused by the mutagenesis created by *p69* transgene insertion rather than by *p69* expression, another *p69* transgenic line P69d was used, which has a weaker phenotype and lower *p69* transcript levels (**Figure 5.1**) than P69c. To eliminate the possibility that the increase of miRNA accumulation was caused by a transgene, 35S-GFP line was used as a transgene control. The results further confirmed that the increase of miRNA accumulation was caused by p69 expression. The accumulation of miR156 (**Figure 5.3 C**, top panel), miR167 (**Figure 5.3 E**, left panel), and miR171 (**Figure 5.3 C**, middle panel) in P69d plants were all higher than those in Wt and 35S-GFP plants (compare lane 3 to lane 1 and 4), but lower than those in P69c plants (compare lane 3 to lane 2), which was correlated with the level of p69 gene expression and the severity of disease symptoms (**Figure 5.1**). There were no differences in miRNA accumulation between Wt and 35S-GFP plants (**Figure 5.3 C**, compare lane1 to lane 4).

The materials used in above experiments were all newly bolted inflorescence. To assay whether miRNA accumulation in other tissues and developmental stages was also enhanced by *p69* expression, 6-day-seedlings and leaves and stems from Wt, p69c and p69d plants were used. The results showed that the accumulations of miR167 in all three

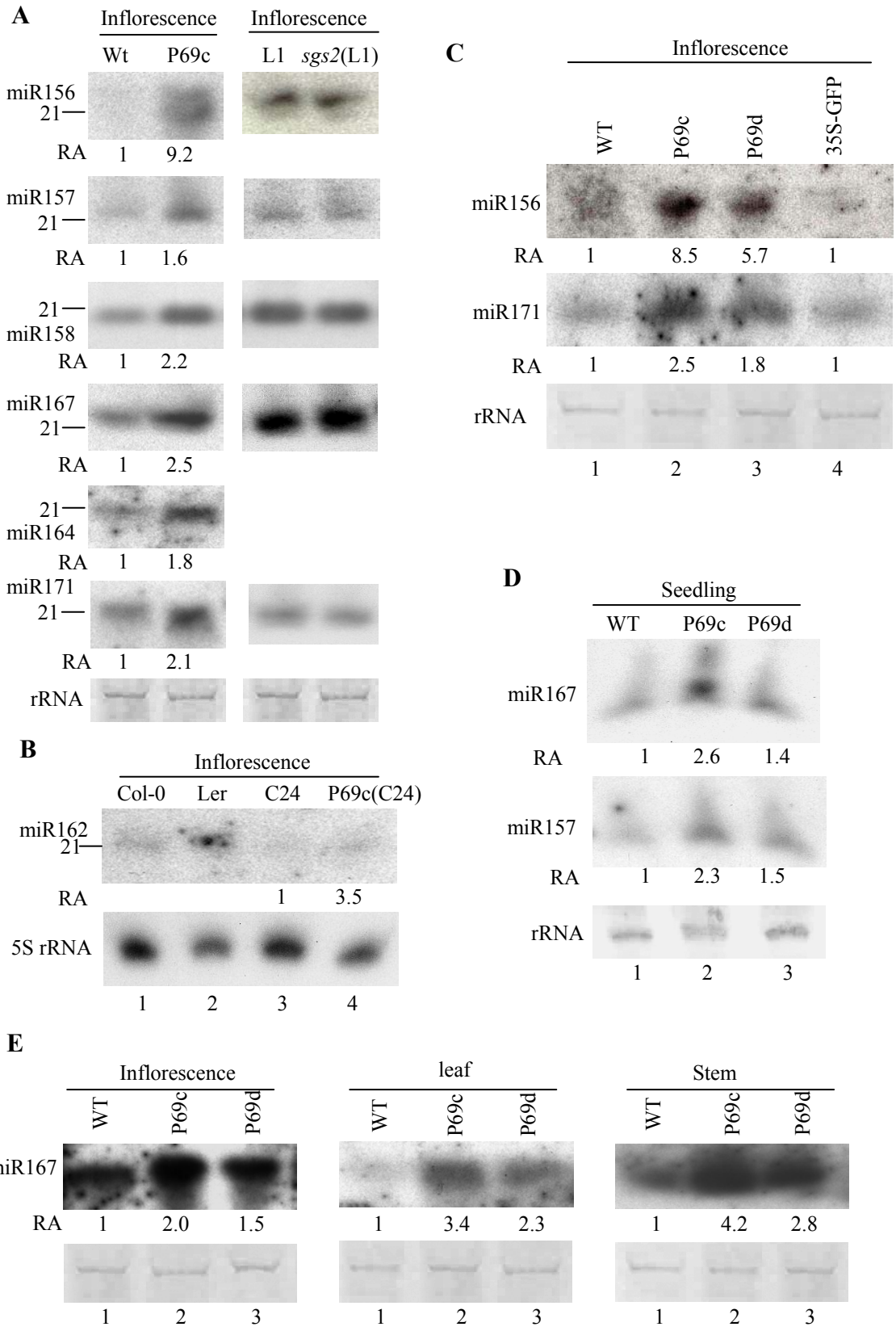

A
Inflorescence
Inflorescence
Wt
P69c
L1
sgs2(L1)
miR156
21
RA 1 9.2
miR157
21
RA 1 1.6
21
miR158
RA 1 2.2
miR167
21
RA 1 2.5
21
miR164
RA 1 1.8
miR171
21
RA 1 2.1
rRNA
B
Inflorescence
Col-0
Ler
C24
P69c(C24)
miR162
21
RA 1 3.5
5S rRNA
1 2 3 4
C
Inflorescence
WT
P69c
P69d
35S-GFP
miR156
RA 1 8.5 5.7 1
miR171
RA 1 2.5 1.8 1
rRNA
1 2 3 4
D
Seedling
WT
P69c
P69d
miR167
RA 1 2.6 1.4
miR157
RA 1 2.3 1.5
rRNA
1 2 3
E
Inflorescence
leaf
Stem
WT
P69c
P69d
miR167
RA 1 2.0 1.5
1 3.4 2.3
1 4.2 2.8
1 2 3
1 2 3
1 2 3

Figure 5.3

Figure 5.3 *p69* enhances miRNA accumulation.

A/B. The accumulation of six miRNAs was enhanced in P69c plants (left panel). But there were no significant differences in miRNA accumulation between L1 and *sgs2* plants (right panel). Fifty μg of total RNA extracted from whole newly bolted inflorescence was loaded in each lane and probed by end labeled DNA oligonucleotides complementary to each of the six miRNAs. The position of a 21-nt RNA marker is indicated. Methylene blue staining of 18S rRNA is shown as a loading control, except for miR162 for which 5S rRNA was used as a loading control. It was noted that the level of miR162 was much lower in Wt ecotype C24 than in either L*er* or Col-0. The RA level for each set of samples is indicated (levels in tissues from Wt were arbitrarily designated as1.0).

C. miRNA accumulation was also increased in P69d line. siRNA gel blot hybridization detected *miR156 and miR171* in two p69 transgenic lines (P69c and P69d), and meanwhile Wt and 35S-GFP transgenic plants were used as controls. The experiments were carried out as described in **A**.

D/E. miRNA accumulation was increased by p69 expression not only in inflorescences but also in seedling, leaves and stems. Seedlings, leaves, and stems from Wt, P69c and P69d were sampled. Thirty ug of total RNA extracted from whole newly bolted inflorescence, leaves and stems respectively, 50 ug of total RNA from seedling was loaded in each lane.

tissues including leaves, stems and 6-day-seedlings and of miR157 in 6-day-seedling were all increased by *p69* expression (**Figure 5.3 E**, middle, right panel; **Figure 5.3 D**). The accumulation of miR167 and miR157 was higher in P69c plants than those of P69d plants, respectively (**Figure 5.3 D**, middle, right panel, and **Figure 5.3 E,** compare lanes 2 to lane 3), which further demonstrated that the accumulation of miRNAs was correlated with p69 transcript level not only in the organ of inflorescence, but also in other tissues.

sgs2/sde1 (RdRP) mutants exhibited little developmental abnormality in contrast to *dcl1* mutants, suggesting that RdRP is not required for miRNA precursor processing. The accumulation of miRNAs should be not affected in *sgs2/sde1* mutants. One experiment was carried out to test the prediction. The results showed no difference in the accumulation of miR156, miR157, miR158, miR167 and miR171 between L1 and the *sgs2* (L1) mutant (**Figure 5.3 A**, right panel), confirming that the cellular RdRP is not involved in miRNA biogenesis. Since *p69* suppression of the siRNA pathway is upstream of dsRNA and resembles the effect of *sgs2/sde1* mutations, the results suggest that the increase of miRNA accumulation in P69c plants is not due to *p69* suppression of the siRNA pathway.

5.3.3 p69 enhances miRNA-mediated cleavage of four target mRNAs

The results in **Figure 5.3 A** showed that miR171 accumulation was increased about 2 times compared to Wt. Since p69 suppresses PTGS by targeting a cellular function leading to dsRNA production, it was predicted that *p69* would not inhibit mRNA cleavage by endogenous miRNAs. Thus, elevated levels of miRNAs in p69 plants may lead to enhanced cleavages of target mRNAs by miRNAs. Initially the cleavage of the

SCARECROW-like 6-IV (*SCL6*-IV) mRNA mediated by miR171 was examined. miR171 mediated cleavage occurs at positions centered in the middle of the miRNA-mRNA duplex so that both the full-length mRNA (SCL6-IVf) and a shorter 3' fragment of ~ 1.3 kilobases (SCL6-IV3') are detected by a 3-proximal probe in flowers of Wt *Arabidopsis* (Llave et al., 2002). *SCL6-IV* belongs to the Scarecrow-like family of putative transcription factors. The members of SCL family control a wide range of developmental processes, including radial patterning in roots and hormone signaling. Northern blot hybridization showed that the cleavage of *SCL6-IV* mRNA indeed occurred in P69c plants as both of the RNA species were detected (**Figure 5.4 A**). Furthermore, there was a decrease in the accumulation of the full-length mRNA and a corresponding increased accumulation of the 3' fragment in P69c plants as compared to Wt plants ((**Figure 5.4 A**). This result suggest that p69 expression did not prevent miR171 cleavage of its mRNA target, and it might actually enhace the process as it was expected from a higher level of miR171 accumulation.

Bioinformatic analysis has identified a number of putative miRNA target genes among the family encoding the SQUAMOSA-promoter binding protein-like genes (*SPL*) (Rhoades et al., 2002). The steady-state levels of three *SPL* mRNAs such as *SPL3, 4* and *9* were examined by Northern blot hybridization. All of these three mRNAs are the predicted targets of miR156. The increase of miR156 accumulation was the most pronounced among all seven miRNAs being tested. These analyses revealed significant decreases in the accumulation of all three *SPL* mRNAs in two independent P69 lines (**Figure 5.4 B**, **C**, **D**, lane 2, 3) as compared to Wt plants (**Figure 5.4 B**, **C**, **D**, lane 1). A decreased accumulation of the *SPL3* and *SPL4* mRNAs was also detected in TYMV

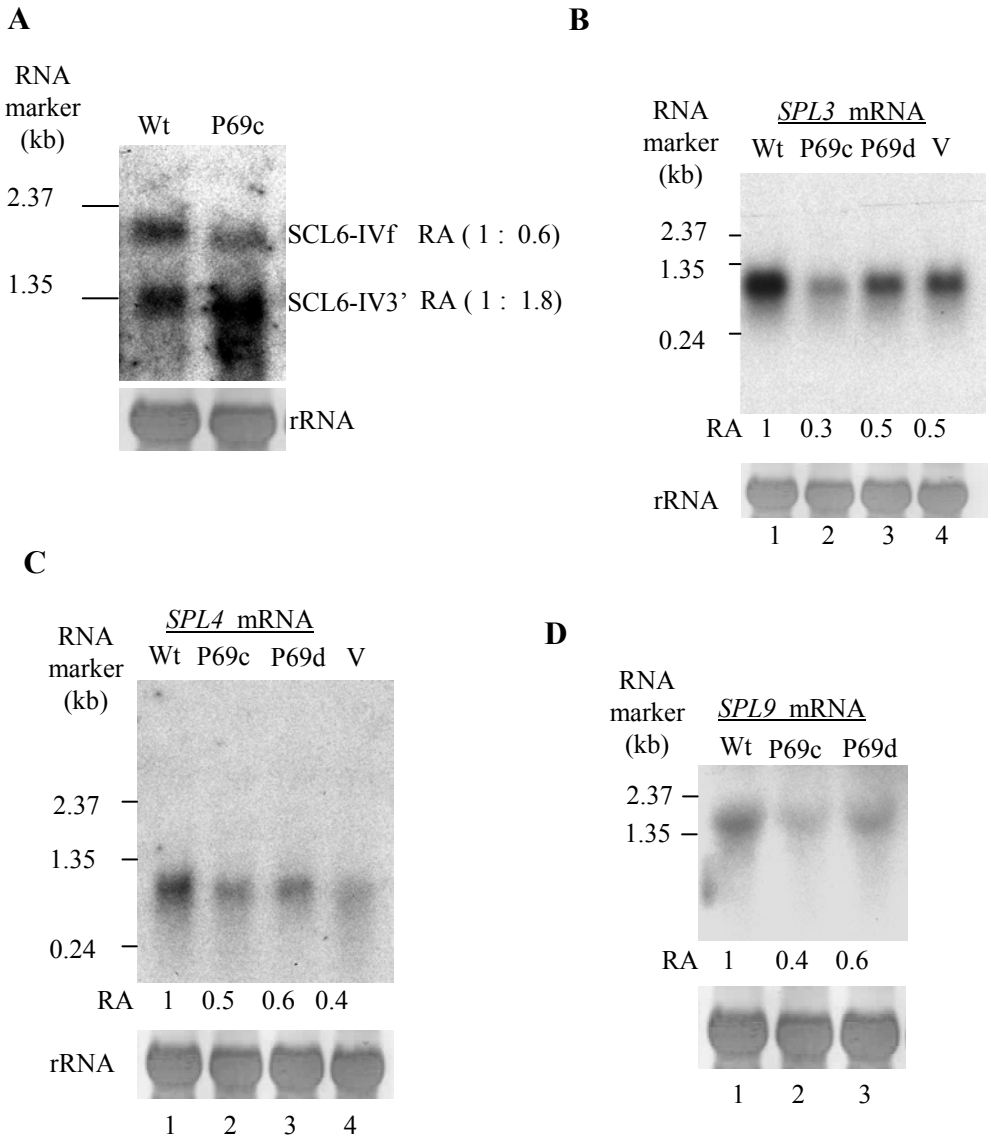

A
RNA marker (kb)
Wt
P69c
2.37
1.35
SCL6-IVf RA (1 : 0.6)
SCL6-IV3' RA (1 : 1.8)
rRNA
B
RNA marker (kb)
SPL3 mRNA
Wt P69c P69d V
2.37
1.35
0.24
RA 1 0.3 0.5 0.5
rRNA
1 2 3 4
C
RNA marker (kb)
SPL4 mRNA
Wt P69c P69d V
2.37
1.35
0.24
RA 1 0.5 0.6 0.4
rRNA
1 2 3 4
D
RNA marker (kb)
SPL9 mRNA
Wt P69c P69d
2.37
1.35
RA 1 0.4 0.6
1 2 3

Figure 5.4

Figure 5.4 *p69* enhanced miRNA-directed cleavage of the mRNAs.

A. *p69* enhanced miR171-directed cleavage of the target *SCL6-IV* mRNAs. RNA gel blot hybridization detected cleavage of *SCL6-IV* mRNA in the presence (right lane) or absence (left lane) of the *p69* transgene. 20μg of total RNAs was loaded in each lane and probed by ^{32}P-labelled DNA specific for the 5' portion of the *SCL6-IV*. The RA level for each set of samples is indicated (levels in tissues from Wt were arbitrarily designated as 1.0).

B. *p69* enhanced miR156-directed cleavage of the target *SPL3* mRNAs. RNA gel blot hybridization detected *SPL3* mRNAs in two p69 transgenic lines (P69c and P69d) and in Wt plants either uninfected or infected systemically with TYMV (V). Twenty μg of total RNAs was loaded in each lane and probed by ^{32}P-labelled DNA specific for the 5' portion *SPL3* mRNAs.

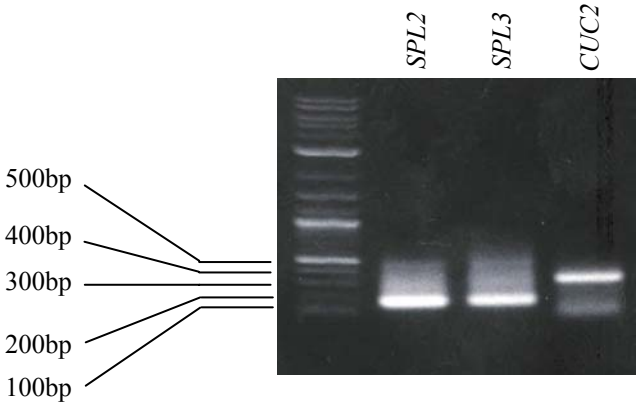
C. p69 enhanced miR156-directed cleavage of the target *SPL4* mRNAs. Samples were used as in **B**. Twenty μg of total RNAs was loaded in each lane and probed by ^{32}P-labelled DNA specific for the 5' portion *SPL4* mRNAs.

D. p69 enhanced miR156-directed cleavage of the target *SPL9* mRNAs. Wt, P69c and P69d plants were sampled. Twenty μg of total RNAs was loaded in each lane and probed by ^{32}P-labelled DNA specific for the 3' portion *SPL9* mRNAs.

infected plants (**Figure 5.4 B**, **C**, lane 4), indicating that enhanced cleavage of *SPL3* and *SPL4* mRNAs may also occur in plants infected with TYMV.

The miR156 has mismatches to the target sites of the three *SPL* mRNAs in contrast to miR171 and siRNAs that are perfectly complementary to their target mRNAs (Llave et al., 2002; Plasterk, 2002). Thus, it is important to determine if the reduced accumulation of these *SPL* mRNAs is due to cleavage at the target sites mediated by miR156. The predicted miR156/*SPL3* mRNA duplex contains one mismatch at the 2nd residue from the 3'-terminus of miR156 in addition to a G:U wobble (Rhoades et al., 2002) (**Figure 5.5 B**). To this end, cDNA corresponding to the predicted 3' fragment of *SPL3* mRNA after cleavage was amplified using the RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) essentially as described (Llave et al., 2002). Primers specific for *SPL2* (At5g43270) and *CUC2* (At5g53950) mRNAs, for which the miRNA cleavage sites were recently mapped (Kasschau et al., 2003), were used as controls. Two rounds of nested PCR using the cDNAs obtained as templates and two sets of RACE with gene-specific primers yielded a discrete band for each mRNA in the predicted size range (**Figure 5.5 A**). Sequencing and alignment of the cloned PCR products with the *SPL3* mRNA identified two cleavage sites in the center of the predicted miR156/mRNA duplex (**Figure 5.5 B**). Similar analyses of the PCR products from *SPL2* and *CUC2* mRNAs confirmed the recently reported results (Kasschau et al., 2003; data not shown). From these findings, it is concluded that cleavage at the predicted duplex of miR156 and *SPL3* mRNA indeed occurred in the P69c plants. Since *SPL3* transcription is developmentally regulated and constitutive over-expression of *SPL3* resulted in early flowering (Cardon et al., 1997), thus these results suggest that the observed reduction in the accumulation of the *SPL3* mRNA

A


SPL2
SPL3
CUC2
500bp
400bp
300bp
200bp
100bp

B

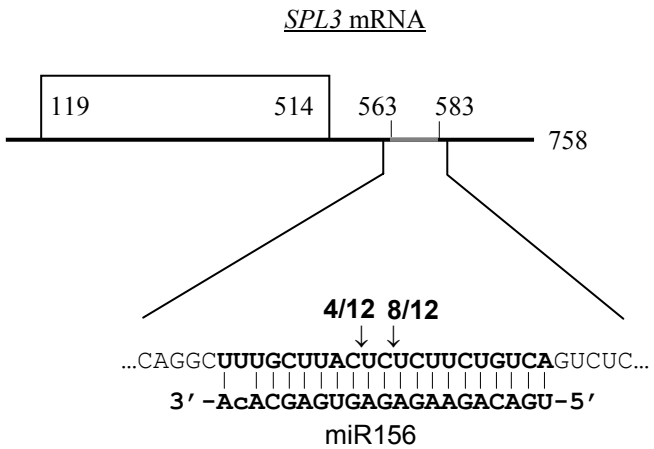

SPL3 mRNA
119
514
563
583
758
4/12
8/12
...CAGGCUUUGCUUACUCUCUUCUGUCAGUCUC...
3′-AcACGAGUGAGAGAAGACAGU-5′
miR156

Figure 5.5

Figure 5.5 Determination of miR156 cleavage sites within *SPL3* mRNA.

A. The 5' RACE method was used to recover the cleavage product for *SPL3* (lane3), meanwhile *SPL2* (lane 2) and *CUC2* (lane 4), for which cleavage sites were published, were used as controls.

B. Full length of *SPL3* mRNA is 758nt and the SPL3 protein is encoded between nt 119-514. The location of the sequence complementary to miR156 is shown in the expanded diagram. Note a U-C mismatch at the left and a G-U wobble at position 7 from the left terminus. In total 12 clones obtained from the 5'-RACE were sequenced and 4 found to be cleaved between AC-U (arrow) and 8 at C-UC (arrow).

and the late flowering phenotype in *p69* transgenic plants may result from an enhanced miR156-mediated inhibition of *SPL3* expression.

5.3.4 p69 increases *DCL1* and *SDE1/SGS2* mRNA accumulation

As the first step to investigate the mechanism of p69-mediated enhancement of both miRNA silencing and IR-RNA induced silencing, the expression of three key genes *SDE1/SGS2, AGO1* and *DCL1* supposedly involved in the PTGS pathway was examined using Northern blotting (**Figure 5.6 A, B**) and /or real time PCR (**Figure 5.6 C**).

Both full length (**Figure 5.6 A**, left panel) and 3' part of mRNAs (**Figure 5.6 A**, right panel) of *DCL1* were at least 3-fold higher in P69c plants than in Wt plants, which was also shown in Real Time PCR analysis (**Figure 5.6 C**). But the full length band of *DCL1* was very weak, when 3' portion probe was used. The reason for this was not clear. Real time PCR analysis also showed an enhanced accumulation of the *SGS2/SDE1* mRNA in P69c plants (**Figure 5.6 C**). However, no significant differences were detected in the accumulation of either *AGO1* or *ß-TUBULIN-1* mRNA between P69c and Wt plants (**Figure 5.6 B** and **C**). This enhanced *DCL1* expression associated with *p69* expression suggests that the increased accumulation of miRNAs and siRNAs detected in *p69*-expressing plants might be a consequence of an accelerated processing from their precursors.

5.4 Discussion

5.4.1 Viral pathogenesis by miRNAs?

The results obtained showed that *p69* gene expression was associated with enhanced accumulation of all of the seven miRNAs examined and with a correspondingly

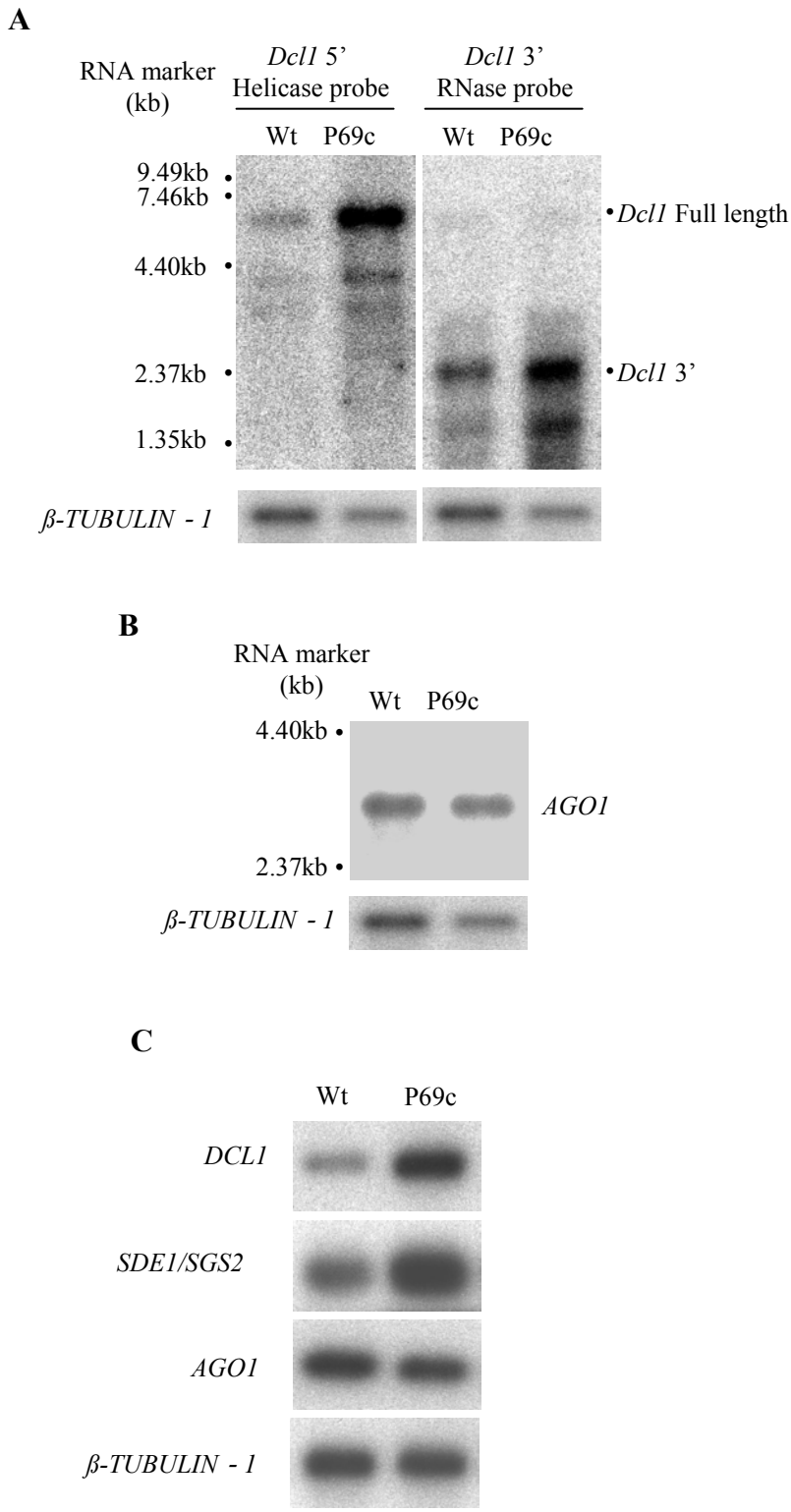

A
RNA marker (kb)
Dcl1 5' Helicase probe
Dcl1 3' RNase probe
Wt
P69c
Wt
P69c
9.49kb
7.46kb
4.40kb
2.37kb
1.35kb
Dcl1 Full length
Dcl1 3'
ß-TUBULIN - 1
B
RNA marker (kb)
Wt
P69c
4.40kb
2.37kb
AGO1
ß-TUBULIN - 1
C
Wt
P69c
DCL1
SDE1/SGS2
AGO1
ß-TUBULIN - 1

Figure 5.6

Figure 5.6 *p69* Increases the expression of *DCL1* and *SDE1/SGS2*.

A. Transcriptional induction of *DCL1* in P69c plants. Two μg mRNAs were loaded in each lane and probed by ^{32}P-labelled DNA corresponding to the 5' helicase domain of *DCL1* (left panel) or 3' RNaseIII domain (right panel). *DCL1* full-length and 3' portion mRNAs of 6.2 and 2.5 kb were detected respectively. The mRNA samples were also analyzed by a probe specific to the *ß-TUBULIN* mRNA as a loading control. mRNA was purified from the total RNA that was isolated from whole plants including leaves, stems, flowers and siliques.

B. No effects on transcription of *AGO1* in P69c plants. ^{32}P-labelled DNA corresponding to the 5'-portion of the *AGO1* coding sequence was used as the probe. AGO1 full-length mRNA of 3.5 kb was detected. The experiment was performed as described as in **A**.

C. Quantitative RT-PCR to examine P69 effects on *RdRP*, *DCL1* and *AGO1* expression.The effect of P69 on expression of the genes *SDE/SGS2* (RdRP), *DCL1*and *AGO1* in the PTGS pathway was investigated via RT-PCR method. The expression of *ß-TUBULIN* gene was used as a control. Amplification was performed by LightCycler Instrument–Real time PCR machine (Roche Applied Science) using the LightCycler-FastStartDNA Master SYBR Green Kit. When PCR was in the log-linear phase before the later plateau phase, the reactions were stopped. 27 cycles was used to amplify *RdRP* gene and 29 cycles were used for *DCL1*, *AGO1, and ß-TUBULIN* genes. PCR products were loaded on an agarose gel for DNA blot hybridization to detect Respective targets. Total RNA was isolated from whole plants including leaves, stems, flowers and siliques.

mRNAs for either cleavage or translational repression (Bartel, 2004, Carrington & Ambros, 2003). Although *p69* could play a role in viral pathogenesis by miRNA-independent mechanisms, an attractive hypothesis is that the disease symptoms/developmental defects associated with *p69* expression in *Arabidopsis* plants represent a consequence of enhanced miRNA-guided inhibition of host gene expression regardless of whether one or both mechanisms are used. For example, the late flowering phenotype of the P69 plants may be in part due to the *p69*-stimulated miR156 knockdown of *SPL3* mRNA, since it is known that constitutive over-expression of *SPL3* results in early flowering (Cardon *et al.*, 1997). Interestingly, the level of *p69* expression is highest in the young leaves systemically infected with TYMV, but declines as infected leaves expand and mature (Bozarth *et al.*, 1992), possibly due to selective proteolysis by the proteasome (Drugeon & Jupin, 2002). Presumably, young tissues are most sensitive to developmental cues transduced by miRNAs, a suggestion consistent with the observation that systemic viral symptoms always appear first in young leaves. Thus, miRNAs may have a novel pathogenic role in the induction of viral diseases.

HC-Pro expression in transgenic plants is known to cause developmental defects (Anandalakshmi et al., 2000). Recent studies show that even though miRNAs accumulate to elevated levels in the presence of HC-Pro, miRNA cleavage of target mRNAs is inhibited in HC-Pro-expressing plants in which for PTGS was induced by either sense- or IR- RNA transgenes (Anandalakshmi et al., 1998, Kasschau & Carrington, 1998, Kasschau et al., 2003, Mallory et al., 2003, Mallory et al., 2002, Xie et al., 2003). Accordingly, it has been proposed that a role of HC-Pro in disease development is to inhibit host developmental pathways that depend on negative regulation by miRNAs (Kasschau et al., 2003). Therefore, the work on p69 of TYMV not only establishes a

distinct mechanism in viral suppression of the RNA silencing antiviral defense, but also suggests an alternative model for the induction of viral diseases by up-regulating the role of miRNAs in the inhibition of host gene expression.

5.4.2 p69 suppression may trigger a negative feedback regulation

It was demonstrated that the accumulation of the mRNA of *DCL1* and *SDE1* was increased by *p69* expression (**Figure 5.6 A, C**), but the accumulation of the mRNA of *AGO1* was not (**Figure 5.6 B, C**). The results also revealed that *p69* expression enhanced the accumulation of all of the seven miRNAs examined (**Figure 5.3**) and of siRNAs derived from the silencing amplicon (**Figure 4.5**) and IR-RNA transgenes (**Figure 4.8, 4.9, 4.10**), and that there was a corresponding decrease in the accumulation of four mRNA targets of miRNAs (**Figure 5.4**). At present, it is not known if *p69* expression leads to increased expression of additional RNA silencing pathway genes such as the homologues of *DCL1* and *SGS2/SDE1* (Finnegan et al., 2003, Yu et al., 2003; Xie et al., 2004) or if any of the p69-induced changes is mechanistically related. Given the known role of *DCL1* in the production of miRNAs (Park et al., 2002, Reinhart et al., 2002), however, it is proposed that the RNA silencing pathway in *Arabidopsis* is under a negative feedback regulation that can be triggered by *p69* suppression or the presence of p69 and that *DCL1* and *SDE1* represent those genes in the pathway that are responsive to this feedback regulation at the level of transcription.

It is likely that enhanced *DCL1* expression will lead to a more efficient processing of precursors, and hence enhanced accumulation of miRNAs and siRNAs. This model is not contradictory to the absence of *GUS* siRNA in L1xP69c plants (**Figure 4.4**) since the

sense-RNA silencing in this system requires production of the dsRNA that is sensitive to *p69* suppression. Interestingly, it was observed that suppression of RNA silencing by the *sgs2* mutation is insufficient to trigger the proposed negative feedback regulation. This is probably because suppression of silencing is more specific in the *sgs2* mutant than in *p69*-expressing plants as one or more of the additional three *SGS2/SDE1* homologues may provide a non-overlapping function to *SGS2/SDE1*(Yu *et al*., 2003). Indeed, *p69* suppression of RNA silencing in GxA plants resulted in higher levels of viral RNAs than the *sde1* mutation did (**Figure 4.5 C**).

Recent research demonstrated that HC-Pro suppressed miRNA-guided cleavage of mRNA targets to trigger developmental defects (Kasschau et al., 2003). It seems contradictary that the levels of both miRNAs and mRNA targets were all enhanced by HC-Pro expression. But a further study showed that a miR162 target sequence was predicted near the middle of *DCL1* mRNA, and miR162-guided cleavage product was identified (Xie et al., 2003). These results indicate that *DCL1* mRNA is subject to negative feedback regulation through the activity of a miRNA. In HC-Pro transgenic plants, *DCL1* RNA was elevated, because HC-Pro inhibits miRNA-guided degradation of target mRNAs. The elevated *DCL1* may drive miRNA precursor processing, which results in over-all increased accumulation of miRNAs. In *p69* transgenic plants, the accumulation of *DCL1* mRNA was also elevated and companied with the enhanced accumulation of miRNAs which is almost the same situation as in HC-Pro transgenic plants. On the other hand, however miR162 also accumulated to a higher level in p69-expressing plants than in Wt plants (**Figure 5.3 B**), which, intriguingly, did not lead to the expected correspondingly lower level of the *DCL1* mRNA (**Figure 5.6**), in contrast to miR156 and miR171. It is not clear if this is because of an overall lower accumulation level of miR162 in C24 than in

Col-0 and L*er* (**Figure 5.3 A, B**)(Xie et al., 2003). Alternatively, miRNA cleavage of mRNAs may be a rate-limiting process so that it becomes ineffective to control mRNA accumulation of those genes that are under transcriptional induction. It is less likely, however, that the increased accumulation of *DCL1* mRNA found in p69-expressing plants is a result of p69-mediated inhibition of miRNA cleavage since mRNA cleavages by miR156 and miR171 were not inhibited and consistent HC-Pro inhibition of miRNA cleavages was observed for ten target mRNAs (Kasschau et al., 2003, Xie et al., 2003). It should be pointed out that a putative role for *DCL1* in elevated levels of miRNAs is based on an assumption that p69 does not inhibit miRNA-mediated translational arrest, which remains to be determined. In this regard, it will be interesting to determine if p69 expression also inhibits host gene expression by miRNA-guided translational repression (Aukerman & Sakai, 2003, Chen, 2003).

PTGS is a natural antiviral defense in plants. Many plant viruses encode PTGS suppressors, suggesting a coevolution of defense and counterdefense. But the virus-host interaction does not stop here. The 2b protein-a suppressor of PTGS expressed from a recombinant tobamovirus can be recognized as an avirulent factor in some plants, initiating a hypersensitive cell-death-response, but not when expressed from its native virus (Li et al., 1999). This poses the possibility that the plant, in response to a virus possessing a mechanism for overcoming the plant's PTGS-based protection (PTGS suppression), uses its hypersensitive response to combat the virus. p69 suppresses sense-RNA silencing, but promotes rather than suppresses IR-RNA and miRNA silencing. It is also like a new round of plant counter defense.

Chapter 6

General Conclusion and Future Prospects

6.1 General conclusion

Small interfering RNAs (siRNAs) and microRNAs (miRNA) are processed by the ribonuclease Dicer from distinct precursors, double-stranded (ds) and hairpin RNAs, although either may guide RNA silencing via a similar complex. The siRNA pathway is antiviral whereas an emerging role for miRNAs is in the control of development. The study in this thesis describes a virulence factor encoded by turnip yellow mosaic virus, p69, that suppresses the siRNA pathway but promotes the miRNA pathway in *Arabidopsis*. p69 suppression of the siRNA pathway occurs upstream of dsRNA and is as effective as genetic mutations in *Arabidopsis* genes involved in dsRNA production. Possibly as a consequence of p69 suppression, p69-expressing plants contained elevated levels of a Dicer mRNA and of miRNAs, as well as a correspondingly enhanced miRNA-guided cleavage of four host mRNAs. Since p69-expressing plants exhibited disease-like symptoms in the absence of viral infection, the findings suggest a novel mechanism for viral virulence by promoting the miRNA-guided inhibition of host gene expression.

Based on these results obtained, a model to describe the mode of action of p69 is proposed (**Figure 6.1)**. p69 suppressor inhibits *SGS1/SDE1* involvement in synthesizing dsRNAs from using aberrant transgene mRNAs. Thus, while virus RNA silencing is sensitive to p69 suppression, silencing induced by IR-RNA and miRNAs is not because the host RdRP branch is not involved. The second aspect of this model suggests that p69

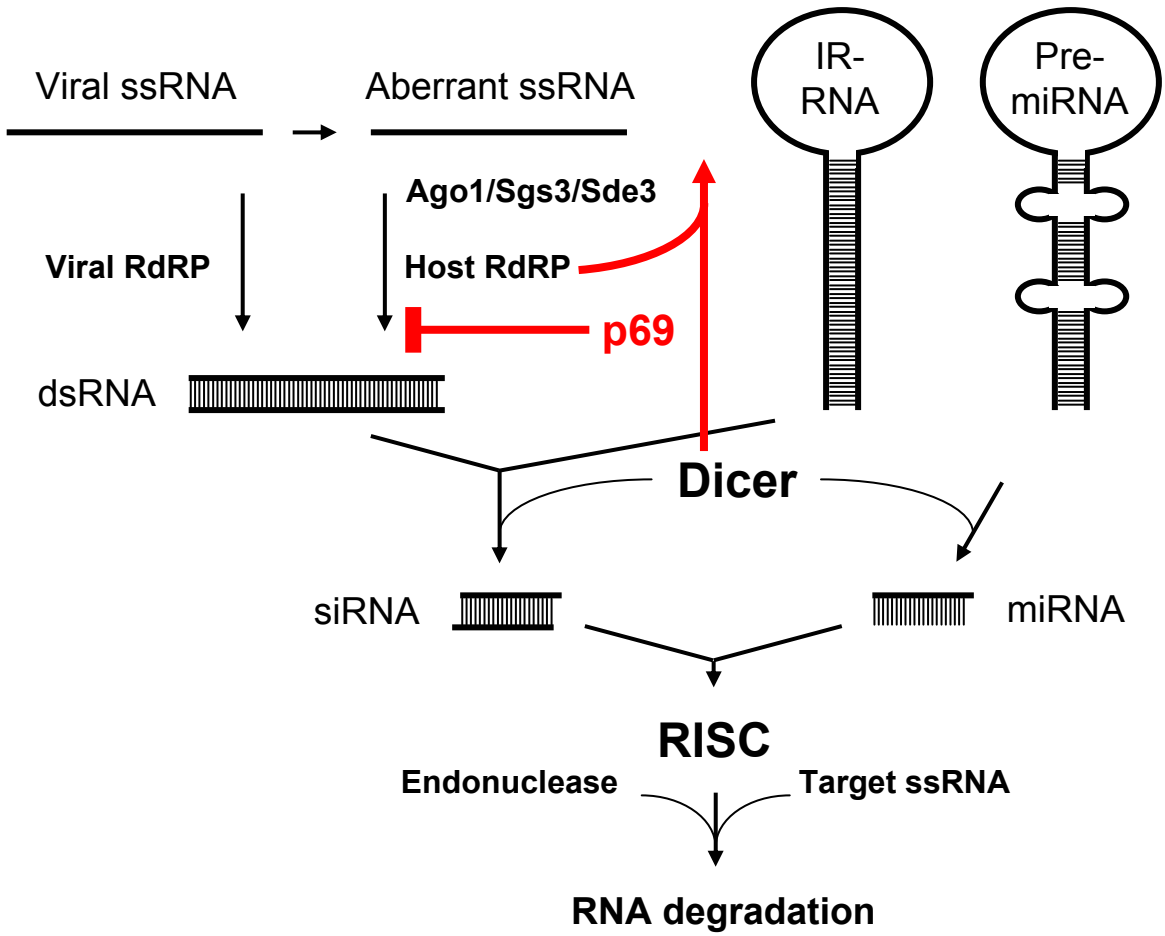

Viral ssRNA
Aberrant ssRNA
IR-
RNA
Pre-
miRNA
Ago1/Sgs3/Sde3
Viral RdRP
Host RdRP
p69
dsRNA
Dicer
siRNA
miRNA
RISC
Endonuclease
Target ssRNA
RNA degradation

Figure 6.1

Figure 6.1 A model for the mode of action of p69.

p69 suppresses PTGS upstream of dsRNA production via the *SGS2/SDE1* (host RdRP)-dependent branch, which triggers a negative feedback regulation of the RNA silencing pathway, leading to transcriptional induction of responsive genes such as *DCL1* (Dicer) and *SGS2/SDE1* and enhanced RNA silencing by IR-RNA and miRNAs that does not involve synthesis of dsRNA through the RdRP branch.

suppression triggers a negative feedback regulation of the RNA silencing pathway, leading to transcriptional induction of responsive genes such as *DCL1* (Dicer) and *SGS2/SDE1*. A prediction of this model is that p69 expression would actually enhance RNA silencing in those systems that do not involve synthesis of dsRNA through the host RdRP-dependent branch. This is consistent with our observations that there were elevated levels of miRNAs and enhanced miRNA-guided cleavages of target mRNAs in p69-expressing plants and that p69 expression resulted in higher accumulation levels of siRNAs from silencing IR-RNA and amplicon transgenes.

6.2 Future Prospects

Besides providing the first description on how p69 interacts with the host PTGS pathway and miRNA regulation pathway, the research in this thesis has also provided an excellent genetic system for further investigation on the mechanisms of PTGS suppression and disease development. Using TYMV-*Arabidopsis* interaction system, we can address the following questions:

1. Do the homologues of TYMV p69 encoded by other tymoviruses such as those that infect *Solanaceae* species suppress PTGS? Is the full-length sequence or part of p69 required for the suppression activity?

2. Does p69 suppression require the direct interaction of any *Arabidopsis* proteins with p69 protein? The Yeast two-hybrid system can be used here to address this question. Are these p69-interacting proteins also required for p69-mediated disease symptoms in *Arabidopsis*? Both of these questions may be addressed by screening for loss of GUS

activity and /or disease symptoms in mutant populations derived from a line homozygous for both p69 and L1 loci.

References

Ambros,V., Bartel,B., Bartel,D.P., Burge,C.B., Carrington,J.C., Chen,X., Dreyfuss,G., Eddy,S.R., Griffiths-Jones,S., Marshall,M., Matzke,M., Ruvkun,G., and Tuschl,T. (2003). A uniform system for microRNA annotation. RNA *9*, 277-279.

Ambros,V., Bartel,B., Bartel,D.P., Burge,C.B., Carrington,J.C., Chen,X., Dreyfuss,G., Eddy,S.R., Griffiths-Jones,S., Marshall,M., Matzke,M., Ruvkun,G., and Tuschl,T. (2003). A uniform system for microRNA annotation. RNA *9*, 277-279.

Anandalakshmi,R., Marathe,R., Ge,X., Herr,J.M., Mau,C., Mallory,A., Pruss,G., Bowman,L., and Vance,V.B. (2000). A calmodulin-related protein that suppresses posttranscriptional gene silencing in plants. Science *290*, 142-144.

Anandalakshmi,R., Pruss,G.J., Ge,X., Marathe,R., Mallory,A.C., Smith,T.H., and Vance,V.B. (1998). A viral suppressor of gene silencing in plants. Proceedings of the National Academy of Sciences of the United States of America *95*, 13079-13084.

Aoki,Y., Cioca,D.P., Oidaira,H., Kamiya,J., and Kiyosawa,K. (2003). RNA interference may be more potent than antisense RNA in human cancer cell lines. Clinical and Experimental Pharmacology and Physiology *30*, 96-102.

Aukerman,M.J. and Sakai,H. (2003). Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes. Plant Cell 15, 2730-2741.

Bahramian,M.B. and Zarbl,H. (1999). Transcriptional and posttranscriptional silencing of rodent alpha 1(I) collagen by a homologous transcriptionally self-silenced transgene. Molecular and Cellular Biology *19*, 274-283.

Banerjee,D. and Slack,F. (2002). Control of developmental timing by small temporal RNAs: a paradigm for RNA-mediated regulation of gene expression. Bioessays *24*, 119-129.

Bartel,B. and Bartel,D.P. (2003). MicroRNAs: At the root of plant development? *Plant Physiology* 132, 709–717.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 116, 281-297.

Baulcombe,D.C. (1996). RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Molecular Biology *32*, 79-88.

Baulcombe,D.C. (1999). Gene silencing: RNA makes RNA makes no protein. Current Biology *9*, R599-R601.

Beclin,C., Berthome,R., Palauqui,J.C., Tepfer,M., and Vaucheret,H. (1998). Infection of tobacco or Arabidopsis plants by CMV counteracts systemic post-transcriptional silencing of nonviral (trans)genes. Virology *252*, 313-317.

Beclin,C., Boutet,S., Waterhouse,P., and Vaucheret,H. (2002). A branched pathway for transgene-induced RNA silencing in plants. Current Biology *12*, 684-688.

Bernstein,E., Caudy,A.A., Hammond,S.M., and Hannon,G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363-366.

Boutet,S., Vazquez,F., Liu,J., Beclin,C., Fagard,M., Gratias,A., Morel,J.B., Crete,P., Chen,X.M., and Vaucheret,H. (2003). Arabidopsis HEN1: A genetic link between endogenous miRNA controlling development and siRNA controlling transgene silencing and virus resistance. Current Biology *13*, 843-848.

Bozarth,C.S., Weiland,J.J., and Dreher,T.W. (1992). Expression of ORF-69 of turnip yellow mosaic virus is necessary for viral spread in plants. Virology *187*, 124-130.

Bransom,K.L., and Dreher, T.W. (1994). Identification of the essential cysteine and histidine residue of the turnip yellow mosaic virus protease. Viology 198, 148-154.

Bransom,K.L., Wallace,S.E., and Dreher,T.W. (1996). Identification of the cleavage site recognized by the turnip yellow mosaic virus protease. Virology *217*, 404-406.

Brigneti,G., Voinnet,O., Li,W.X., Ji,L.H., Ding,S.W., and Baulcombe,D.C. (1998). Viral pathogenicity determinants are suppressors of transgene silencing in Nicotiana benthamiana. EMBO Journal *17*, 6739-6746.

Brown,S.J., Mahaffey,J.P., Lorenzen,M.D., Denell,R.E., and Mahaffey,J.W. (1999). Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. Evolution & Development *1*, 11-15.

Canady,M.A., Day,J., and McPherson,A. (1995). Preliminary-X-Ray Diffraction Analysis of Crystals of Turnip Yellow Mosaic-Virus (Tymv). Proteins-Structure Function and Genetics *21*, 78-81.

Canady,M.A., Larson,S.B., Day,J., and McPherson,A. (1996). Crystal structure of turnip yellow mosaic virus. Nature Structural Biology *3*, 771-781.

Cardon,G.H., Hohmann,S., Nettesheim,K., Saedler,H., and Huijser,P. (1997). Functional analysis of the Arabidopsis thaliana SBP-box gene SPL3: a novel gene involved in the floral transition. Plant J. *12*, 367-377.

Carmell,M.A., Xuan,Z.Y., Zhang,M.Q., and Hannon,G.J. (2002). The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes & Development *16*, 2733-2742.

Carrington,J.C., Kasschau,K.D., and Johansen,L.K. (2001). Activation and suppression of RNA silencing by plant viruses. Virology *281*, 1-5.

Carrington,J.C. and Whitham,S.A. (1998). Viral invasion and host defense: strategies and counter-strategies. Current Opinion in Plant Biology *1*, 336-341.

Carthew,R.W. (2001). Gene silencing by double-stranded RNA. Current Opinion in Cell Biology *13*, 244-248.

Catalanotto,C., Azzalin,G., Macino,G., and Cogoni,C. (2000). Transcription - Gene silencing in worms and fungi. Nature *404*, 245.

Caudy,A.A., Ketting,R.F., Hammond,S.M., Denli,A.M., Bathoorn,A.M.P., Tops,B.B.J., Silva,J.M., Myers,M.M., Hannon,G.J., and Plasterk,R.H.A. (2003). A micrococcal nuclease homologue in RNAi effector complexes. Nature 425, 411-414.

Caudy,A.A., Myers,M.M., Hannon,G.J., and Hammond,S.M. (2002). Fragile X-related protein and VIG associate with the RNA interference machinery. Genes & Development 16,2491-2496.

Chen,X. (2003). A microRNA as a translational repressor of *APETALA2* in *Arabidopsis* flower development. Published online July 31 2003; 10.1126/science.1088060

Chicas,A. and Macino,G. (2001). Characteristics of post-transcriptional gene silencing. EMBO Reports *2*, 992-996.

Chuang,C.F. and Meyerowitz,E.M. (2000). Specific and heritable genetic interference by double-stranded RNA in Arabidopsis thaliana. Proceedings of the National Academy of Sciences of the United States of America *97*, 4985-4990.

Clough,S.J. and Bent,A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. *16*, 735-743.

Coburn,G.A. and Cullen,B.R. (2002a). Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. Journal of Virology *76*, 9225-9231.

Cogoni,C. and Macino,G. (1999a). Homology-dependent gene silencing in plants and fungi: a number of variations on the same theme. Current Opinion in Microbiology *2*, 657-662.

Cogoni,C. and Macino,G. (1999b). Posttranscriptional gene silencing in Neurospora by a RecQ DNA helicase. Science *286*, 2342-2344.

Cogoni,C. and Macino,G. (2000). Post-transcriptional gene silencing across kingdoms. Current Opinion in Genetics & Development *10*, 638-643.

Cogoni,C., Romano,N., and Macino,G. (1994). Suppression of Gene-Expression by Homologous Transgenes. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology *65*, 205-209.

Covey,S.N., Alkaff,N.S., Langara,A., and Turner,D.S. (1997). Plants combat infection by gene silencing. Nature *385*, 781-782.

Dalmay,T., Hamilton,A., Mueller,E., and Baulcombe,D.C. (2000a). Potato virus X amplicons in arabidopsis mediate genetic and epigenetic gene silencing. Plant Cell *12*, 369-379.

Dalmay,T., Hamilton,A., Rudd,S., Angell,S., and Baulcombe,D.C. (2000b). An RNA-dependent RNA polymerase gene in Arabidopsis is required for posttranscriptional gene silencing mediated by a transgene but not by a virus. Cell *101*, 543-553.

Dalmay,T., Horsefield,R., Braunstein,T.H., and Baulcombe,D.C. (2001). SDE3 encodes an RNA helicase required for posttranscriptional gene silencing in Arabidopsis. EMBO Journal *20*, 2069-2077.

de Wit,T., Grosveld,F., and Drabek,D. (2002). The tomato RNA-directed RNA polymerase has no effect on gene silencing by RNA interference in transgenic mice. Transgenic Research *11*, 305-310.

Ding,S.W. (2000). RNA silencing. Current Opinion in Biotechnology *11*, 152-156.

Ding,S.W., Li,W.X., and Symons,R.H. (1995). A Novel Naturally-Occurring Hybrid Gene Encoded by A Plant Rna Virus Facilitates Long-Distance Virus Movement. EMBO Journal *14*, 5762-5772.

Doi,N., Zenno,S., Ueda,R., Ohki-Hamazaki,H., Ui-Tei,K., and Saigo,K. (2003). Short-interfering-RNA-mediated gene silencing in mammalian cells requires dicer and eIF2C translation initiation factors. Current Biology *13*, 41-46.

Dostie,J.E., Mourelatos,Z., Yang,M., Sharma,A., and Dreyfuss,G. (2003). Numerous microRNPs in neuronal cells containing novel microRNAs. RNA-A Publication of the RNA Society *9*, 180-186.

Dougherty,W.G. and Parks,T.D. (1995). Transgenes and Gene Suppression - Telling Us Something New. Current Opinion in Cell Biology *7*, 399-405.

Drugeon,G. and Jupin,I. (2002). Stability in vitro of the 69K movement protein of Turnip yellow mosaic virus is regulated by the ubiquitin-mediated proteasome pathway. J. Gen. Virol. *83*, 3187-3197.

Dudley,N.R., Labbe,J.C., and Goldstein,B. (2002). Using RNA interference to identify genes required for RNA interference. Proceedings of the National Academy of Sciences of the United States of America *99*, 4191-4196.

Elbashir,S.M., Lendeckel,W., and Tuschl,T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes & Development *15*, 188-200.

Elmayan,T., Balzergue,S., Beon,F., Bourdon,V., Daubremet,J., Guenet,Y., Mourrain,P., Palauqui,J.C., Vernhettes,S., Vialle,T., Wostrikoff,K., and Vaucheret,H. (1998). Arabidopsis mutants impaired in cosuppression. Plant Cell *10*, 1747-1757.

Elmayan,T. and Vaucheret,H. (1996). Expression of single copies of a strongly expressed 35S transgene can be silenced post-transcriptionally. Plant Journal *9*, 787-797.

Emery,J.F., Floyd,S.K., Alvarez,J., Eshed,Y., Hawker,N.P., Izhaki,A., Baum,S.F. and Bowman,J.L. (2003). Radial patterning of Arabidopsis shoots by class III HD-ZIP and KANADI genes. Curr. Biol. 13, 6877-6888.

Enright,A.J., John,B., Gaul,U., Tuschl., Sander,C., and Marks,D.S. (2003). MicroRNA targets in Drosophila. Genome Biol. 5, R1.

Errampalli,D., Patton,D., Castle,L., Mickelson,L., Hansen,K., Schnall,J., Feldmann,K., and Meinke,D. (1991). Embryonic Lethals and T-DNA Insertional Mutagenesis in Arabidopsis. Plant Cell *3*, 149-157.

Fagard,M., Boutet,S., Morel,J.B., Bellini,C., and Vaucheret,H. (2000). AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants,

quelling in fungi, and RNA interference in animals. Proceedings of the National Academy of Sciences of the United States of America *97*, 11650-11654.

Finnegan,E.J., Margis,R., and Waterhouse,P.M. (2003). Posttranscriptional gene silencing is not compromised in the Arabidopsis CARPEL FACTORY (DICER-LIKE1) mutant, a homolog of dicer-1 from Drosophila. Current Biology *13*, 236-240.

Fire,A. (1999). RNA-triggered gene silencing. Trends in Genetics *15*, 358-363.

Fire,A., Xu,S.Q., Montgomery,M.K., Kostas,S.A., Driver,S.E., and Mello,C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Foster,T.M., Lough,T.J., Emerson,S.J., Lee,R.H., Bowman,J.L., Forster,R.L., and Lucas,W.J. (2002) A Surveillance System Regulates Selective Entry of RNA into the Shoot Apex. The Plant Cell 14, 1497-1508.

Gorbalenya,A.E., Koonin,E.V., Donchenko,A.P., and Blinov,V.M. (1989) Two related superfamilies of putative helicases involved in replication, recombination, repair, and expression of DNA and RNA genomes. Nucleic Acids Res. 17:4713–4730.

Grishok,A., Pasquinelli,A.E., Conte,D., Li,N., Parrish,S., Ha,I., Baillie,D.L., Fire,A., Ruvkun,G., and Mello,C.C. (2001a). Genes and mechanisms related to RNA interference

regulate expression of the small temporal RNAs that control C-elegans developmental timing. Cell *106*, 23-34.

Grishok,A., Tabara,H., and Mello,C.C. (2000). Genetic requirements for inheritance of RNAi in C-elegans. Science *287*, 2494-2497.

Guo,H.S. and Ding,S.W. (2002d). A viral protein inhibits the long range signaling activity of the gene silencing signal. EMBO Journal *21*, 398-407.

Guo,S. and Kemphues,K.J. (1995). Par-1, A Gene Required for Establishing Polarity in C-Elegans Embryos, Encodes A Putative Ser/Thr Kinase That Is Asymmetrically Distributed. Cell *81*, 611-620.

Ha,I., Wightman,B., and Ruvkun,G. (1996). A bulged lin-4/lin-14 RNA duplex is sufficient for Caenorhabditis elegans lin-14 temporal gradient formation. Genes & Development *10*, 3041-3050.

Hamilton,A., Voinnet,O., Chappell,L., and Baulcombe,D. (2002). Two classes of short interfering RNA in RNA silencing. EMBO J. *21*, 4671-4679.

Hamilton,A.J. and Baulcombe,D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science *286*, 950-952.

Hammond,S.M., Bernstein,E., Beach,D., and Hannon,G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.

Hammond,S.M., Boettcher,S., Caudy,A.A., Kobayashi,R., and Hannon,G.J. (2001). Argonaute2, a link between genetic and biochemical analyses of RNAi. Science *293*, 1146-1150.

Hannon,G.J. (2002). RNA interference. Nature *418*, 244-251.

Hernandez-Hernandez,V., Fernandez,J., Cardona,A., Romero,R., and Bueno,D. (2001). Selective inhibition of gene expression by RNAi in chick embryos in ovo. International Journal of Developmental Biology *45*, S99-S100.

Hutvagner,G., McLachlan,J., Pasquinelli,A.E., Balint,E., Tuschl,T., and Zamore,P.D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834-838.

Hutvagner,G. and Zamore,P.D. (2002a). A microRNA in a multiple-turnover RNAi enzyme complex. Science *297*, 2056-2060.

Hutvagner,G. and Zamore,P.D. (2002b). RNAi: nature abhors a double-strand. Current Opinion in Genetics & Development *12*, 225-232.

Jacobsen,S.E., Running,M.P., and Meyerowitz,E.M. (1999). Disruption of an RNA helicase/RNAse III gene in Arabidopsis causes unregulated cell division in floral meristems. Development *126*, 5231-5243.

Ji,L.H. and Ding,S.W. (2001). The suppressor of transgene RNA silencing encoded by Cucumber mosaic virus interferes with salicylic acid-mediated virus resistance
JI2001. Molecular Plant-Microbe Interactions *14*, 715-724.

Jones,L. (2002). Revealing micro-RNAs in plants. Trends in Plant Science *7*, 473-475.

Jones,L., Hamilton,A.J., Voinnet,O., Thomas,C.L., Maule,A.J., and Baulcombe,D.C. (1999). RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell *11*, 2291-2301.

Jones,L., Ratcliff,F., and Baulcombe,D.F. (2001). RNA-directed transcriptional gene silencing in plants can be inherited independently of the RNA trigger and requires Met1 for maintenance. Current Biology *11*, 747-757.

Kadare,G., Rozanov,M. and Haenni,A.L. (1995). Expression of the turnip yellow mosaic virus proteinase in *Escherichia coli* and determination of the cleavage site within the 206-kDa protein. J. Gen. Virol. 76:2853–2857.

Kasschau,K.D. and Carrington,J.C. (1998). A counterdefensive strategy of plant viruses: Suppression of posttranscriptional gene silencing. Cell *95*, 461-470.

Kasschau,K.D., Xie,Z., Allen,E., Llave,C., Chapman,E.J., Krizan,K.A., and Carrington,J.C. (2003). P1/HC-Pro, a Viral Suppressor of RNA Silencing, Interferes with Arabidopsis Development and miRNA Function. Dev. Cell *4*, 205-217.

Keese,P., Mackenzie,A., and Gibbs,A. (1989). Nucleotide sequence of the genome of an Australian isolate of turnip yellow mosaic tymovirus. Virology *172*, 536-546.

Ketting,R.F., Fischer,S.E.J., Bernstein,E., Sijen,T., Hannon,G.J., and Plasterk,R.H.A. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C-elegans. Genes & Development *15*, 2654-2659.

Ketting,R.F., Haverkamp,T.H.A., van Luenen,H.G.A.M., and Plasterk,R.H.A. (1999). mut-7 of C-elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell *99*, 133-141.

Khvorova,A., Reynolds,A., and Jayasena,S.D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell 115, 209-216.

Knight,S.W. and Bass,B.L. (2001). A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science *293*, 2269-2271.

Korneev,S.A., Kemenes,I., Straub,V., Staras,K., Korneeva,E.I., Kemenes,G.R., Benjamin,P.R., and O'Shea,M. (2002). Suppression of nitric oxide (NO)-dependent behavior by double-stranded RNA-mediated silencing of a neuronal NO synthase gene. Journal of Neuroscience *22*, art-RC227.

Kamer,G., and Argos,P. (1984) Primary structural comparison of RNAdependent polymerases from plant, animal and bacterial viruses. Nucleic Acids Res. 12:7269–7282.

Lagos-Quintana,M., Rauhut,R., Lendeckel,W., and Tuschl,T. (2001). Identification of novel genes coding for small expressed RNAs. Science *294*, 853-858.

Lau,N.C., Lim,L.P., Weinstein,E.G., and Bartel,D.P. (2001). An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science *294*, 858-862.

Lee,R.C. and Ambros,V. (2001). An extensive class of small RNAs in Caenorhabditis elegans. Science *294*, 862-864.

Lee,R.C., Feinbaum,R.L., and Ambros,V. (1993). The C-Elegans Heterochronic Gene Lin-4 Encodes Small RNAs with Antisense Complementarity to Lin-14. Cell *75*, 843-854.

Lee,Y., Ahn,C., Han,J., Choi,H., Kim,J., Yim,J., Lee,J., Provost,P., Rådmark,O., Kim,S., and Kim,V.N. (2003). The nuclear RNase III Drosha initiates microRNAs processing. Nature *425, 415-419*.

Lee,Y., Joen,K., Lee,J., Kim,S., and Kim,V.N. (2002). MicroRNA maturation: stepwise processing and subcellullar localization. The EMBO Journal 21, 4663-4670.

Lewis,B.P., Shih, I., Jones-Rhoades,M.W., Bartel,D.P., and Burge,C.B. (2003). Prediction of mammalian microRNAs targets. Cell 115, 787-798.

Li,H.W., Li,W.X., and Ding,S.W. (2002). Induction and suppression of RNA silencing by an animal virus. Science *296*, 1319-1321.

Li,H.W., Lucy,A.P., Guo,H.S., Li,W.X., Ji,L.H., Wong,S.M., and Ding,S.W. (1999). Strong host resistance targeted against a viral suppressor of the plant gene silencing defence mechanism. EMBO Journal *18*, 2683-2691.

Li,W.X., Li,H., Lu,R., Li,F., Dus,M., Atkinson,P., Brydon,E.W.A., Johnson,K.L., Garcia-Sastre,A., Ball,L.A., Palese,P., and Ding,S.W. (2004). Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. PNAS 101, 1350-1355.

Li,W.X. and Ding,S.W. (2001). Viral suppressors of RNA silencing. Current Opinion in Biotechnology *12*, 150-154.

Lim,L.P., Lau,N.C., Weistein,E.G., Abdelhakim,A., Yekta,S., Rhoades,M.W., Burge,C.B., and bartel,D.P. (2003a). The microRNAs of *Caenorhabditis elegans*. Genes Dev. 17, 991-1008.

Lim,L.P., Glasner,M.E., Yekta,S., Burge,C.B., and Bartel,D.P. (2003b). Vertebrate microRNAs genes. Science 299, 1540.

Lindbo,J.A., Silvarosales,L., and Dougherty,W.G. (1993). Pathogen Derived Resistance to Potyviruses - Working, But Why. Seminars in Virology *4*, 369-379.

Lipardi,C., Wei,Q., Paterson,B.M. (2001), RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell 107(3):297-307.

Llave,C., Kasschau,K.D., and Carrington,J.C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. Proceedings of the National Academy of Sciences of the United States of America *97*, 13401-13406.

Llave,C., Kasschau,K.D., Rector,M.A., and Carrington,J.C. (2002a). Endogenous and silencing-associated small RNAs in plants. Plant Cell *14*, 1605-1619.

Llave,C., Xie,Z.X., Kasschau,K.D., and Carrington,J.C. (2002b). Cleavage of Scarecrow-like mRNA targets directed by a class of Arabidopsis miRNA. Science *297*, 2053-2056.

Lucy,A.P., Guo,H.S., Li,W.X., and Ding,S.W. (2000). Suppression of post-transcriptional gene silencing by a plant viral protein localized in the nucleus. EMBO Journal *19*, 1672-1680.

Lund,E., Guttinger,S., Calado,A., Dahlberg,J.E., and Kutay,U. (2004). Nuclear export of nicroRNA precursors. Science 297, 2053-2056.

Makeyev,E.V. and Bamford,D.H. (2002). Cellular RNA-dependent RNA polymerase involved in posttranscriptional gene silencing has two distinct activity modes. Molecular Cell *10*, 1417-1427.

Mallory,A.C., Ely,L., Smith,T.H., Marathe,R., Anandalakshmi,R., Fagard,M., Vaucheret,H., Pruss,G., Bowman,L., and Vance,V.B. (2001). HC-Pro suppression of transgene silencing eliminates the small RNAs but not transgene methylation or the mobile signal. Plant Cell *13*, 571-583.

Mallory,A.C., Mlotshwa,S., Bowman,L.H., and Vance,V. (2003). The Capacity of transgenic tobacco to send a systemic RNA silencing signal depends on the nature of the inducing transgene locus. Plant J. In press.

Mallory,A.C., Reinhart,B.J., Bartel,D., Vance,V.B., and Bowman,L.H. (2002). A viral suppressor of RNA silencing differentially regulates the accumulation of short interfering RNAs and micro-RNAs in tobacco. Proceedings of the National Academy of Sciences of the United States of America *99*, 15228-15233.

Martinez,J., Patkaniowska,A., Urlaub,H., Luhrmann,R., and Tuschl,T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110, 563-574.

Matzke,M., Matzke,A.J.M., and Kooter,J.M. (2001a). RNA: Guiding gene silencing. Science *293*, 1080-1083.

Matzke,M.A. (2002). Gene silencing mechanisms illuminate new pathways of disease resistance. Transgenic Research *11*, 637-638.

Matzke,M.A., Matzke,A.J.M., Pruss,G.J., and Vance,V.B. (2001). RNA-based silencing strategies in plants. Current Opinion in Genetics & Development *11*, 221-227.

McElver,J., Tzafrir,I., Aux,G., Rogers,R., Ashby,C., Smith,K., Thomas,C., Schetter,A., Zhou,Q., Cushman,M.A., Tossberg,J., Nickle,T., Levin,J.Z., Law,M., Meinke,D., and Patton,D. (2001). Insertional mutagenesis of genes required for seed development in Arabidopsis thaliana. Genetics *159*, 1751-1763.

Mette,M.F., Matzke,A.J.M., and Matzke,M.A. (2001). Resistance of RNA-mediated TGS to HC-Pro, a viral suppressor of PTGS, suggests alternative pathways for dsRNA processing. Current Biology *11*, 1119-1123.

Mitter,N., Sulistyowati,E., and Dietzgen,R.G. (2003). Cucumber mosaic virus infection transiently breaks dsRNA-induced transgenic immunity to Potato virus Y in tobacco. Molecular Plant Microbe Interaction, 16, 936-944.

Mlotshwa,S., Voinnet,O., Mette,M.F., Matzke,M., Vaucheret,H., Ding,S.W., Pruss,G., and Vance,V.B. (2002). RNA silencing and the mobile silencing signal. *Plant Cell,* **14,** S289-S301

Morch,M.D., Boyer,J.C., and Haenni,A.L. (1988). Overlapping open reading frames revealed by complete nucleotide sequencing of turnip yellow mosaic virus genomic RNA. Nucleic Acids Res. *16*, 6157-6173.

Morel,J.B., Godon,C., Mourrain,P., Beclin,C., Boutet,S., Feuerbach,F., Proux,F., and Vaucheret,H. (2002). Fertile hypomorphic ARGONAUTE (ago1) mutants impaired in post-transcriptional gene silencing and virus resistance. Plant Cell *14*, 629-639.

Morel,J.B., Mourrain,P., Beclin,C., and Vaucheret,H. (2000). DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in Arabidopsis. Current Biology *10*, 1591-1594.

Moss,E.G., Lee,R.C., and Ambros,V. (1997). The cold shock domain protein LIN-28 controls developmental timing in C-elegans and is regulated by the lin-4 RNA. Cell *88*, 637-646.

Mourelatos,Z., Dostie,J., Paushkin,S., Sharma,A., Charroux,B., Abel,L., Rappsilber,J., Mann,M., and Dreyfuss,G. (2002). miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes & Development *16*, 720-728.

Mourrain,P., Beclin,C., Elmayan,T., Feuerbach,F., Godon,C., Morel,J.B., Jouette,D., Lacombe,A.M., Nikic,S., Picault,N., Remoue,K., Sanial,M., Vo,T.A., and Vaucheret,H. (2000). Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell *101*, 533-542.

Napoli,C., Lemieux,C., and Jorgensen,R. (1990). Introduction of A Chimeric Chalcone Synthase Gene Into Petunia Results in Reversible Co-Suppression of Homologous Genes in Trans. Plant Cell *2*, 279-289.

Nykanen,A., Haley,B., and Zamore,P.D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell *107*, 309-321.

Olsen,P.H. and Ambros,V. (1999). The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Developmental Biology *216*, 671-680.

Page,M.F., Carr,B., Anders,K.R., Grimson,A., and Anderson,P. (1999). SMG-2 is a phosphorylated protein required for mRNA surveillance in Caenorhabditis elegans and related to Upf1p of yeast. Molecular and Cellular Biology *19*, 5943-5951.

Pal-Bhadra,M., Bhadra,U., and Birchler,J.A. (2002). RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in Drosophila. Molecular Cell *9*, 315-327.

Palatnik,J.F., Allen,E., Wu,X., Schommer,C., Schwab,R., Carrington, and Weigel,D. (2003). Control of leaf morphogenesis by miRNAs. Nature 425, 257-263.

Palauqui,J.C. and Balzergue,S. (1999). Activation of systemic acquired silencing by localised introduction of DNA. Current Biology *9*, 59-66.

Palauqui,J.C., Elmayan,T., Pollien,J.M., and Vaucheret,H. (1997). Systemic acquired silencing: Transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO Journal *16*, 4738-4745.

Park,W., Li,J.J., Song,R.T., Messing,J., and Chen,X.M. (2002a). CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Current Biology *12*, 1484-1495.

Park,W.S., Miyano-Kurosaki,N., Hayafune,M., Nakajima,E., Matsuzaki,T., Shimada,F., and Takaku,H. (2002b). Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. Nucleic Acids Research *30*, 4830-4835.

Parrish,S. and Fire,A. (2001). Distinct roles for RDE-1 and RDE-4 during RNA interference in Caenorhabditis elegans. RNA-A Publication of the RNA Society *7*, 1397-1402.

Pasquinelli,A.E., Reinhart,B.J., Slack,F., Martindale,M.Q., Kuroda,M.I., Maller,B., Hayward,D.C., Ball,E.E., Degnan,B., Muller,P., Spring,J., Srinivasan,A., Fishman,M., Finnerty,J., Corbo,J., Levine,M., Leahy,P., Davidson,E., and Ruvkun,G. (2000). Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature *408*, 86-89.

Palatinik,J.F., Allen,E., Wu,X., Schommer,C., Schwab,J., Carrington,J.C., and Weigel,D. (2003) Control of leaf morphogenesis by microRNAs. Nature *425*, 257-263.

Papp,I. Mette,M.F., Aufsatz,W., Daxinger,L., Schauer,S.E., Ray,A., van der Winden,J., Matzke,M., and Matzke,A.J. (2003). Evidence for nuclear processing of plant microRNAs and short interfering RNA precursors. Plant Physiol. 132, 1382-1390.

Pasquinelli,A.E. and Ruvkun,G. (2002). Control of developmental timing by microRNAs and their targets. Annual Review of Cell and Developmental Biology *18*, 495-513.

Paszkowski,J. and Whitham,S.A. (2001). Gene silencing and DNA methylation processes. Current Opinion in Plant Biology *4*, 123-129.

Pfeffer,S., Dunoyer,P., Heim,F., Richards,K.E., Jonard,G., and Ziegler-Graff,V. (2002). P0 of beet Western yellows virus is a suppressor of posttranscriptional gene silencing. J. Virol. *76*, 6815-6824.

Plasterk,R.H.A. (2002). RNA silencing: The genome's immune system. Science *296*, 1263-1265.

Plasterk,R.H.A. and Ketting,R.F. (2000). The silence of the genes. Current Opinion in Genetics & Development *10*, 562-567.

Powers,A.M., Kamrud,K.I., Olson,K.E., Higgs,S., Carlson,J.O., and Beaty,B.J. (1996). Molecularly engineered resistance to California serogroup virus replication in mosquito cells and mosquitoes. Proceedings of the National Academy of Sciences of the United States of America *93*, 4187-4191.

Prod'homme,D., Le Panse,S., Drugeon,G., and Jupin.I. (2001). Detection and subcellular localization of the turnip yellow mosaic virus 66K replication protein in infected cells. Virology 281:88–101.

Pruss,G., Ge,X., Shi,X.M., Carrington,J.C., and Vance,V.B. (1997). Plant viral synergism: The potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. Plant Cell *9*, 859-868.

Qu,F., Ren,T., and Morris,T.J. (2003). The coat protein of turnip crinkle virus suppresses posttranscriptional gene silencing at an early initiation step. Journal of Virology *77*, 511-522.

Ratcliff,F., Harrison,B.D., and Baulcombe,D.C. (1997). A similarity between viral defense and gene silencing in plants. Science *276*, 1558-1560.

Ratcliff,F., Martin-Hernandez,A.M., and Baulcombe,D.C. (2001). Tobacco rattle virus as a vector for analysis of gene function by silencing. Plant Journal *25*, 237-245.

Ratcliff,F.G., MacFarlane,S.A., and Baulcombe,D.C. (1999). Gene silencing without DNA: RNA-mediated cross-protection between viruses. Plant Cell *11*, 1207-1215.

Ray,S., Golden,T., and Ray,A. (1996). Maternal effects of the short integument mutation on embryo development in Arabidopsis. Developmental Biology *180*, 365-369.

Reinhart,B.J., Slack,F.J., Basson,M., Pasquinelli,A.E., Bettinger,J.C., Rougvie,A.E., Horvitz,H.R., and Ruvkun,G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature *403*, 901-906.

Reinhart,B.J., Weinstein,E.G., Rhoades,M.W., Bartel,B., and Bartel,D.P. (2002). MicroRNAs in plants. Genes & Development *16*, 1616-1626.

Rhoades,M.W., Reinhart,B.J., Lim,L.P., Burge,C.B., Bartel,B., and Bartel,D.P. (2002). Prediction of plant microRNA targets. Cell *110*, 513-520.

Rozanov,M., Koonin,E. and Gorbalenya,A. (1992). Conservation of the putative methyltransferase domain: a hallmark of the 'Sindbis-like' supergroup of positive-strand RNA viruses. J. Gen. Virol. 73:2129–2134.

Ruiz,M.T., Voinnet,O., and Baulcombe,D.C. (1998). Initiation and maintenance of virus-induced gene silencing. Plant Cell *10*, 937-946.

Ruvkun,G. (2001). Molecular biology - Glimpses of a tiny RNA world. Science *294*, 797-799.

Schauer,S.E., Jacobsen,S.E., Meinke,D.W., and Ray,A. (2002). DICER-LIKE1: blind men and elephants in Arabidopsis development. Trends in Plant Science *7*, 487-491.

Schwarz,D.S., Hutvagner,G., Haley,B., and Zamore,P.D. (2002). Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Molecular Cell *10*, 537-548.

Schwarz,D.S., Hutvagner,G., Du,T., Xu,Z., Aronin,N., and Zamore,P.D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell 115, 199-208.

Sharp,P.A. (2001). RNA interference - 2001. Genes & Development *15*, 485-490.

Sijen,T., Fleenor,J., Simmer,F., Thijssen,K.L., Parrish,S., Timmons,L., Plasterk,R.H.A., and Fire,A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465-476.

Silhavy,D., Molnar,A., Lucioli,A., Szittya,G., Hornyik,C., Tavazza,M., and Burgyan,J. (2002). A viral protein suppresses RNA silencing and binds silencing-generated, 21-to 25-nucleotide double-stranded RNAs. EMBO Journal *21*, 3070-3080.

Skotnicki,M.L., Mackenzie,A.M., and Gibbs,A.J. (1992). Turnip yellow mosaic virus variants produced from DNA clones encoding their genomes. Arch. Virol. *127*, 25-35.

Skuzeski,J.M., Bozarth,C.S., and Dreher,T.W. (1996). The turnip yellow mosaic virus tRNA-like structure cannot be replaced by generic tRNA-like elements or by heterologous

3' untranslated regions known to enhance mRNA expression and stability. Journal of Virology *70*, 2107-2115.

Slack,F.J., Basson,M., Liu,Z.C., Ambros,V., Horvitz,H.R., and Ruvkun,G. (2000). The lin-41 RBCC gene acts in the C-elegans heterochronic pathway between the let-7 regulatory RNA and the LIN-29 transcription factor. Molecular Cell *5*, 659-669.

Smardon,A., Spoerke,J.M., Stacey,S.C., Klein,M.E., Mackin,N., and Maine,E.M. (2000). EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in C-elegans. Current Biology *10*, 169-178.

Smith,N.A., Singh,S.P., Wang,M.B., Stoutjesdijk,P.A., Green,A.G., and Waterhouse,P.M. (2000). Gene expression - Total silencing by intron-spliced hairpin RNAs. Nature *407*, 319-320.

Soderman,E., Mattsson,J., Engstrom,P. (1996). The Arabidopsis homeobox gene ATHB-7 is induced by water deficit and by abscisic acid. Plant Journal 10(2):375-81.

Stark,A., Brennecke,J., Russell,R.B., and Cohen,S.M. (2003). Identification of Drosophila microRNAs targets. PLOS Biol. 1, E60.

Stein,P., Svoboda,P., Anger,M., and Schultz,R.M. (2003). RNAi: Mammalian oocytes do it without RNA-dependent RNA polymerase. RNA-A Publication of the RNA Society *9*, 187-192.

Szittya, G., Molnar, A., Silhavy, D., Hornyik, C. & Burgyan, J. (2002). Short defective interfering RNAs of tombusviruses are not targeted but trigger post-transcriptional gene silencing against their helper virus. *Plant Cell* **14**, 359-372.

Tabara,H., Grishok,A., and Mello,C.C. (1998). RNAi in C-elegans: Soaking in the genome sequence. Science *282*, 430-431.

Tabara,H., Sarkissian,M., Kelly,W.G., Fleenor,J., Grishok,A., Timmons,L., Fire,A., and Mello,C.C. (1999). The rde-1 gene, RNA interference, and transposon silencing in C-elegans. Cell *99*, 123-132.

Tabara,H., Yigit,E., Siomi,H., and Mello,C.C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExX-box helicase to direct RNAi in C-elegans. Cell *109*, 861-871.

Takeda,A., Sugiyama,K., Nagano,H., Mori,M., Kaido,M., Mise,K., Tsuda,S., and Okuno,T. (2002a). Identification of a novel RNA silencing suppressor, NSs protein of Tomato spotted wilt virus. FEBS Lett. *532*, 75-79.

Tang,G.L., Reinhart,B.J., Bartel,D.P., and Zamore,P.D. (2003). A biochemical framework for RNA silencing in plants. Genes & Development *17*, 49-63.

Tsai,C.H. and Dreher,T.W. (1993). Increased viral yield and symptom severity result from a single amino acid substitution in the turnip yellow mosaic virus movement protein. Mol. Plant Microbe Interact. *6*, 268-273.

Tuschl,T., Zamore,P.D., Lehmann,R., Bartel,D.P., and Sharp,P.A. (1999). Targeted mRNA degradation by double-stranded RNA in vitro. Genes & Development *13*, 3191-3197.

Vaistij,F.E., Jones,L., and Baulcombe,D.C. (2002). Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. Plant Cell *14*, 857-867.

Vance,V. and Vaucheret,H. (2001). RNA silencing in plants - Defense and counterdefense. Science *292*, 2277-2280.

Vance,V.B., Berger,P.H., Carrington,J.C., Hunt,A.G., and Shi,X.M. (1995). 5'-Proximal Potyviral Sequences Mediate Potato-Virus-X Potyviral Synergistic Disease in Transgenic Tobacco. Virology *206*, 583-590.

Van der krol,A.R., Mur,L.A., Beld,M., Mol,J.N.M., and Stuitje,A.R. (1990). Flavonoid Genes in Petunia - Addition of A Limited Number of Gene Copies May Lead to A Suppression of Gene-Expression. Plant Cell *2*, 291-299.

Vargason,J.M., Szittya,G., Burgyan,J., and Hall,T.M.T. (2003). Size selective recognition siRNA by RNA silencing suppressor. Cell 115, 799-811.

Vaucheret,H., Beclin,C., and Fagard,M. (2001). Post-transcriptional gene silencing in plants. Journal of Cell Science *114*, 3083-3091.

Vaucheret,H. and Fagard,M. (2001). Transcriptional gene silencing in plants: targets, inducers and regulators. Trends in Genetics *17*, 29-35.

Voinnet,O. (2001). RNA silencing as a plant immune system against viruses. Trends in Genetics *17*, 449-459.

Voinnet,O. and Baulcombe,D.C. (1997). Systemic signalling in gene silencing. Nature *389*, 553.

Voinnet,O., Lederer,C., and Baulcombe,D.C. (2000). A viral movement protein prevents spread of the gene silencing signal in Nicotiana benthamiana. Cell *103*, 157-167.

Voinnet,O., Pinto,Y.M., and Baulcombe,D.C. (1999). Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. Proceedings of the National Academy of Sciences of the United States of America *96*, 14147-14152.

Voinnet,O., Vain,P., Angell,S., and Baulcombe,D.C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell *95*, 177-187.

Wang,M.B. and Waterhouse,P.M. (2002). Application of gene silencing in plants. Current Opinion in Plant Biology *5*, 146-150.

Waterhouse,P.M., Graham,M.W., and Wang,M.B. (1998), Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA Proc Natl Acad Sci 95(23):13959-64.

Waterhouse,P.M., Wang,M.B., and Lough,T. (2001). Gene silencing as an adaptive defence against viruses. Nature *411*, 834-842.

Weiland,J.J., and Dreher,T.W. (1989). Infectious TYMV RNA from cloned cDNA: effects in vitro and in vivo of point substitutions in the initiation codons of two extensively overlapping ORFs. Nucleic Acids Res. 17:4675–4687.

Weiland,J.J., and Dreher,T.W. (1993). Cis-preferential replication of the turnip yellow mosaic virus RNA genome. Proc. Natl. Acad. Sci. USA 90: 6095–6099.

Wightman,B., Ha,I., and Ruvkun,G. (1993). Posttranscriptional Regulation of the Heterochronic Gene Lin-14 by Lin-4 Mediates Temporal Pattern-Formation in C-Elegans. Cell *75*, 855-862.

Williams R.W., Rubin G.M. (2002) ARGONAUTE1 is required for efficient RNA interference in Drosophila embryos. PNAS 99(10):6889-94

Winston,W.M., Molodowitch,C., and Hunter,C.P. (2002). Systemic RNAi in C-elegans requires the putative transmembrane protein SID-1. Science *295*, 2456-2459.

Wu-Scharf,D., Jeong,B.R., Zhang,C.M., and Cerutti,H. (2000). Transgene and transposon silencing in Chlamydomonas reinhardtii by a DEAH-Box RNA helicase. Science *290*, 1159-1162.

Xie,Z.X., Kasschau,K.D., and Carrington,J.C. (2003). Negative feedback regulation of Dicer-Like1 in Arabidopsis by microRNA-guided mRNA degradation. Current Biology *13*, 784-789.

Xie,Z., Johansen,L.K., Gustafson,A.M., Kasschau,K.D., Lellis,A.D., Zilberman,D., Jacobsen,S.E., Carrington,J.C. (2004). Genetic and Functional Diversification of Small RNA Pathways in Plants. PLoS Biol. 24,2(5):E104

Xu,L., Liu,F., Lechner,E., Genschik,P., Crosby,W.L., Ma,H., Peng,W., Huang,D., and Xie,D. (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in Arabidopsis. Plant Cell *14*, 1919-1935.

Yamamoto,T., Omoto,S., Mizuguchi,M., Mizukami,H., Okuyama,H., Okada,N., Saksena,N.K., Brisibe,E.A., Otake,K., and Fujii,Y.R. (2002). Double-stranded nef RNA interferes with human immunodeficiency virus type 1 replication. Microbiology and Immunology *46*, 809-817.

Ye,K., Malimina,L., and Patel,D.J. (2003). Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature 426, 874-878.

Yi,R., Qin,Y., Macara,I.G., and Cullen,B.R. (2003). Exportin-5 mediates the nuclear export of pre-microRNAs and the short hairpin RNAs. Genes Dev. 17, 3011-3016.

Yu,D.Q., Fan,B.F., MacFarlane,S.A., and Chen,Z.X. (2003). Analysis of the involvement of an inducible Arabidopsis RNA-dependent RNA polymerase in antiviral defense. Molecular Plant-Microbe Interactions *16*, 206-216.

Zamore,P.D., Tuschl,T., Sharp,P.A., and Bartel,D.P. (2000). RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25-33.

Zeng,Y., and Cullen,B.R. (2003). Sequence requirements for microRNAs processing and function in human cells. RNA 9, 112-123.

Zilberman,D., Cao,X.F., and Jacobsen,S.E. (2003). ARGONAUTE4 control of locus-specific siRNA accumulation and DNA and histone methylation. Science *299*, 716-719.

Zuo,J.R. and Chua,N.H. (2000). Chemical-inducible systems for regulated expression of plant genes. Current Opinion in Biotechnology *11*, 146-151.